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PROCEEDINGS OF THE ROYAL SOCIETY.

SECTION B.—BIOLOGICAL SCIENCES.

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Diffusion and Chemical Reaction Velocity as Joint Factors in determining the Rate of Uptake of Oxygen and Carbon Monoxide by the Red Blood Corpuscle.

By F. J. W. ROUGHTON, Fellow of Trinity College, Cambridge.

(Communicated by J. Barcroft, F.R.S.—Received October 10, 1931.)

GENERAL INTRODUCTION.

In a paper on this subject published four years ago, Hartridge and Roughton (1927) described some preliminary experiments upon the rate of uptake of oxygen and carbon monoxide by the red blood corpuscle, the observations being made by means of their rapid reaction velocity technique (Hartridge and Roughton, 1922–1927). The general principles of the method were as follows.

Through one lead of the apparatus a suspension of reduced corpuscles in saline was forced into the mixing chamber, whilst through the other lead was forced a solution of oxygen (or carbon monoxide) in saline. The two fluids mixed in the mixing chamber within 0.001 second or less and then travelled down the observation tube. Determination of the percentage of oxyhæmoglobin (or carboxyhæmoglobin) in the moving fluid at various cross sections of the observation tube was made by means of the reversion spectroscope, these measurements, together with a knowledge of the rate of flow of the fluid down the observation tube, giving the necessary data for plotting the rate of uptake of O_2 or CO by the corpuscles against time. The most interesting feature of the results was the much slower uptake of O_2 by hæmoglobin in the intact corpuscle as compared with the rate of uptake of O_2 by hæmoglobin in

laked solution as previously recorded by Hartridge and Roughton (1925). In the corpuscle experiments the time scale had to be expressed in hundredths of a second instead of in thousandths of a second as in the hæmoglobin solution experiments (*vide* fig. 2 of Hartridge and Roughton, 1927). Confirmatory results by a somewhat different technique have been obtained lately by Dirken and Mook (1931). These will be referred to again later.

Observations of this kind seem to be of interest and application in at least two different connections.

(A) First of all there is the question, which is primarily of biophysical and biochemical rather than physiological significance, namely, the mechanism of the interchange of gas between the corpuscle and its surrounding solution. The possible factors involved have already been considered elsewhere, for Hartridge and Roughton (1927) in seeking to account for the much slower rate of oxygen uptake by the corpuscles than by hæmoglobin solution, suggested that the discrepancy between the rates of the two processes might be due to any of the following four factors acting singly or in combination :—

(i) The existence of appreciable concentration gradients in the suspending fluid.

The O_2 which is acquired by the corpuscle must come, in the first instance, from the layers of suspending fluid adjacent to the corpuscles. The loss suffered by such layers will then be replenished by diffusion and by mixing with more distant layers. The latter two processes may be sufficiently slow to limit, or at any rate to affect, the rate of the actual process under investigation.

(ii) The existence of an appreciable concentration gradient through the corpuscle membrane.

We have as yet no certain quantitative information either as to the thickness or the diffusion coefficient of the corpuscle membrane. There is consequently a possibility that the O_2 on the outside of the membrane may dam up, until there is a sufficient gradient through the membrane to enable O_2 to enter the corpuscle at a rate equal to that at which it is removed by combination with the hæmoglobin.

(iii) The existence of an appreciable concentration gradient inside the corpuscle.

The O_2 which first enters the corpuscle will combine with the peripheral layer of Hb molecules. The next instalments of O_2 will have to diffuse through the outer saturated layers in order to find Hb molecules which are still uncombined with O_2 . It is possible that such diffusion may, as in (ii), be so slow as to produce an appreciable gradient of dissolved O_2 inside the corpuscle.

Any of factors (i), (ii), (iii) acting singly or in combination would involve a lower value of the average concentration of dissolved O_2 in immediate contact with the Hb molecules, when the latter are present in the corpuscle instead of in solution. There still, however, remains

(iv) The possibility that the kinetics of the reaction between O_2 and Hb in the corpuscle may be so altered that even at the same average concentration of dissolved O_2 the rate of increase of per cent. O_2 Hb may be 10 times smaller in the case of the corpuscle than in the case of the Hb solution.

Hartridge and Roughton (1927) concluded, as a result of several control experiments in which both the violence and quality of the motion of the suspending fluid were varied, that no appreciable concentration gradients could exist in the fluid outside the corpuscle. This conclusion is fundamental to the mathematical analysis given in Section II below if this is to be applicable to the theoretical interpretation of experiments on the rate of inflow of O_2 or CO into the red blood corpuscle. It is therefore desirable to consider the matter more fully than was done in the original paper.

Hartridge and Roughton's most important control was the one (*vide* fig. 3 of their paper) in which they showed that a threefold variation in the rate of flow caused no detectable change in the rate of inflow of O_2 into the corpuscle. There seem to be only two possible* interpretations of this result :—

- (a) That at both rates of flow, the suspending fluid between the corpuscles was practically stationary and that O_2 was supplied to the corpuscle entirely by diffusion without the aid of fluid stirring.
- (b) That at both rates of flow, the stirring of the suspending fluid was so thorough that the stagnant films of fluid adhering to the corpuscle were of negligible thickness, and hence the concentration of dissolved gas in all parts of the suspending fluid, *including that in immediate contact with the walls of the corpuscles*, must have been practically constant throughout.

If the actual state of affairs was intermediate between the two extremes (a) and (b), it is impossible to understand how a threefold increase in the rate of fluid movement could have failed to have caused more efficient stirring of the suspending fluid and hence an increase in the observed rate of inflow.

Possibility (a) can be ruled out as follows :—

* The Brownian movements of the corpuscles can be shown by calculation to be too small to be of any importance in this connection.

If diffusion alone is operating in the suspending fluid, the pressure gradient per centimetre necessary to account for the observed rate of inflow during the first half of the process can be calculated on the same lines as the calculations given on pp. 7-8. It is found to be about 10^6 mm. Hg* per centimetre. But since the maximum pressure of dissolved O_2 in the outside fluid only amounted to 70 mm., no appreciable supplies of O_2 could have arrived, merely through diffusion, from points distant greater than $70/10^6$ cm., *i.e.*, 7×10^{-5} cm. Now the average distance between the corpuscles in these experiments was about 1.5×10^{-3} cm., *i.e.*, some 20 times greater, and since during the first half of the process about half of the total dissolved O_2 in the surrounding fluid is drawn into the corpuscle, it is clear that by far the greater amount of the O_2 which flowed into the corpuscle must have come from distances many times greater than 7×10^{-5} cm. Thus diffusion alone in the suspending fluid is quite unable to account for the observed rate of inflow, and the only possibility left is (b)—namely, that the stirring in the outside fluid was so efficient that the concentration of dissolved O_2 in the surrounding fluid was sensibly constant everywhere including even the actual periphery of the corpuscles.

The purpose of the present paper is to analyse these corpuscular factors further. As the result of theoretical consideration, a method has been evolved which *may* lead to the solution of the whole problem, but since this will certainly involve lengthy and difficult experimentation, and hence great delay, it seems advisable in the meantime to give an account of the results obtained up to the present, together with a short note about future developments. This preliminary work has involved the theoretical investigation of a system in which diffusion and chemical reaction velocity are both limiting factors. Such must be a condition of common physiological occurrence, though, so far as the writer knows, not hitherto worked out before.

(B) From the more purely physiological point of view, the rate of uptake of O_2 by, and/or the rate of escape of O_2 from the red blood corpuscle is of obvious significance in investigating the acquisition of oxygen by the blood in the lung, and the unloading of oxygen from the blood in the tissues. In regard to the former of these problems, it has already been pointed out in the previous paper (Hartridge and Roughton, 1927) that the experiments there described indicated that the values of the diffusion constant of the lung, as calculated by the Bohr-Krogh method, were too low. Since then a further interest has been given to the matter by the suggestion of Bock, Dill, Edwards, Henderson and Talbot

* O_2 concentration is here measured in the units of partial pressure defined on p. 5.

(1929) that slowness of distribution of oxygen within the red blood corpuscle might account for their finding that the pressure of oxygen in the arterial is often, even under normal conditions, markedly lower than the pressure of oxygen in the alveolar air. The position here reached is the same as in the first case, namely, that theoretical reconnaissance has also brought the research a considerable stage further, but shows that further experimental work is again necessary if these problems are to be solved.

SECTION I.

Theoretical consideration of the factors involved in the uptake and release of O_2 and CO from the red blood corpuscle.

In the problems dealt with below, the concentration of dissolved gas (O_2 or CO) will be expressed not in the physical units of gram per cubic centimetre or molecule per litre, but in the units often used by physiologists, viz., partial pressure of dissolved gas, the latter being measured in millimetres Hg. To avoid misunderstanding, the nature and advantage of this unit for the present work must now be stated.

In gas phases partial pressure has its ordinary meaning, but in liquid phases it is best defined by the following equation, viz.,

$$pa = c,$$

where

p = partial pressure of dissolved gas in millimetres Hg.

a = volume of dissolved gas (expressed in cubic centimetres N.T.P.) which is dissolved in 1 c.c. of the liquid when the latter is in equilibrium with a gas phase containing a partial pressure of the gas = 1 mm. Hg [a is the usual solubility coefficient divided by 760].

c = concentration of dissolved gas (expressed in cubic centimetres dissolved gas N.T.P. per cubic centimetre solution).

The partial pressure is then equal to the "vapour pressure of the dissolved gas, or to G. N. Lewis's "fugacity" if the gas is ideal. This unit is specially convenient when gas is diffusing from one phase (*e.g.*, the gas space in the lungs or the blood plasma) to another phase (*e.g.*, the inside of the blood corpuscle). For the gradient causing diffusion must be proportional to the difference of partial pressure between the two phases, whatever their respective solubility coefficients may be, whereas it would only be proportional to the concentration difference between the two phases expressed in grams per cubic centimetre if

the solubility coefficient of the two phases happened to be the same. The latter is far from being so in the present instances.

(A) *Experimental Data so far Available.*

At the outset it will be useful to summarise the experimental results recorded by Hartridge and Roughton (1927). These are given in Table I. Unfortunately, in the case of the processes $O_2 + Hb \rightarrow O_2Hb$, $CO + Hb \rightarrow COHb$ there were considerable variations in the rates observed when blood preparations from different sheep were used, and it is only in the case of the last two processes $O_2Hb \rightarrow O_2 + Hb$, and $COHb + O_2 \rightarrow CO + O_2Hb$ (the data of which have not hitherto been published) that this factor was controlled.

Table I.

	Reaction in corpuscle.		Reaction in solution.		Ratio.	
	Number of experiments.	Time to reach 50 per cent. in seconds.	Number of experiments.	Time to reach 50 per cent. in seconds.	Corpuscle rate.	Solution rate.
* $O_2 + Hb \rightarrow O_2Hb$	6	0.03 to 0.08 (Average 0.05)	6	0.0023 to 0.008 (Average 0.004)	1	12
* $CO + Hb \rightarrow COHb$...	1	0.14	6	0.02 to 0.08 average	1	3.5
$O_2Hb \rightarrow O_2 + Hb$	1	0.4	1	0.1	1	4
† $COHb \rightarrow CO + O_2Hb$	1	60	1	60	1	1

Sheep's blood used throughout.

* Initial pressure of dissolved gas equals 75 mm. Hg approximately. Rate found to be roughly proportional to pressure of dissolved O_2 . Temperature 15° to 20° C. p_H about 7.4, corpuscles suspended in saline.

† By shaking COHb solution or CO saturated corpuscle suspension violently with O_2 gas and measuring the per cent. of COHb by the reversion spectroscopie after various times of shaking (Hartridge and Roughton, 1926).

Recently measurements of the rates of $O_2 + Hb \rightarrow O_2Hb$, and of $O_2Hb \rightarrow O_2 + Hb$ in the blood corpuscle have been described by Dirken and Mook (1931) using a modified form of the reaction velocity apparatus with an ultra-filtration technique for following the O_2 content. These experiments were all done on ox corpuscles at a temperature of about 15° C., p_H about 7.4. Reference to their Table III shows that the time for $O_2Hb \rightarrow O_2 + Hb$ to go to 50 per cent. of completion is about 0.45 second, which is very near to the

figure of 0.4 second given in our Table I. In their experiments upon $O_2 + Hb \rightarrow O_2Hb$ (given in their Table II), which they carried out by mixing reduced blood with a concentrated solution of oxyhæmoglobin followed by measurements of the saturation of the surrounding Hb solution with oxygen at various times after mixture, they obtained rates of uptake of O_2 of the order of 10 times smaller than those given in our Table I. This discrepancy is readily accounted for. As soon as O_2 migrates from the surrounding Hb solution into the corpuscle, the per cent. saturation of the surrounding Hb must fall, so that the pressure of dissolved O_2 in the surrounding Hb solution, which at most could only equal the value which would be in equilibrium with the per cent. O_2Hb in the solution, must fall likewise. Even at 90 per cent. O_2Hb (which according to their Table II the outside solution reaches in about 0.1 second) the O_2 pressure in equilibrium would only amount in the case of average ox blood at p_H 7.4, 15° C. to about 5 mm., which is of the order of only one-tenth the pressure of dissolved O_2 prevailing in our experiments. It will be remembered that we found that the rate of uptake of O_2 by the corpuscle was roughly proportional to the pressure of dissolved O_2 in the surrounding solution. and taking this factor into account and using an average O_2 dissociation curve over the rest of Dirken's and Mook's points, it is found that their agreement with our results is just about as close as in the case of $O_2Hb \rightarrow \dot{O}_2 + Hb$. In the latter case, the factor just discussed does come in also, but to a far smaller extent than in Dirken and Mook's experiments on $O_2 + Hb \rightarrow O_2Hb$.

Table I of the present paper shows that the slower the rate of the chemical action, the smaller is the discrepancy between the rate in solution and in corpuscle until finally in the case of the slowest reaction, *i.e.*, the dissociation of CO-hæmoglobin, the discrepancy is inappreciable. This is just what would be expected if the chemical kinetics of the reactions in the corpuscle were much the same as in solution, the influence of diffusion (whether through the corpuscle membrane or through the finite thickness of the corpuscle or both) naturally becoming less and less as the reaction itself becomes slower. This conclusion, however, cannot be warranted without a detailed consideration of the two diffusion processes.

(B) *The Diffusion Through the Corpuscle Membrane.*

In order to determine the rôle of this factor it is necessary therefore to know—

- (i) The area of the corpuscle membrane = A sq. cm.
- (ii) The thickness of the corpuscle membrane = b_m cm.

- (iii) The diffusion constant of the corpuscle membrane to dissolved O_2 or $CO = D_m$.

Then the volume of dissolved gas (cubic centimetres N.T.P.) diffusing through the corpuscle membrane per second if the partial pressure of dissolved gas on one side is p mm. Hg, and on the other side p' mm. Hg

$$= (p - p') A \cdot \frac{D_m}{b_m}, \quad (I)$$

where D_m , the diffusion coefficient = cubic centimetres of gas N.T.P. diffusing through 1 sq. cm. per second under a gradient of 1 mm. partial pressure per centimetre.

With this equation one could then calculate the pressure difference between either side of the membrane which would be necessary to supply the corpuscle with its observed rate of uptake. If this calculated pressure difference should be small in comparison with the average pressure difference between the interior of the corpuscle and the surrounding fluid, then clearly the influence of diffusion through the membrane on the rate of the whole process, would also be small and *vice versa* if the calculated pressure difference should be relatively large.

Now A may be taken the same as the area of the whole corpuscle which may be obtained from Ponder's careful measurements. According to this author, the sheep's corpuscles are biconcave discs of diameter about 6μ , minimum thickness at the centre 1.4μ , maximum thickness at the periphery 1.7μ .

Unfortunately, however, there is no certain knowledge either about b_m or D_m . As regards b_m , it is true that several electrical estimates, in which the corpuscles are regarded as condensers (the membrane playing the part of the dielectric), give figures of the order of 10^{-6} to 10^{-7} cm. thick, which would mean a layer of 1 to 4 molecules thick if composed of protein or orientated fatty molecules (as suggested by Gorter and Creordel). But this evidence does not seem strong enough to the writer to justify his using it for quantitative calculations at this stage.

As regards D_m , the situation is much worse. The corpuscle membrane is normally permeable to anions and not to cations, and on the analogy of Northrop's (1929) work on dried collodion membranes, which are the only artificial membranes known to the writer with a selective ionic permeability, the corpuscle membrane would be expected to have diffusion constants much less than that of water. The function of the fat in the membrane is, however, very ambiguous, for if orientated in condensed films it would reduce diffusion,

as in Rideal's (1925) experiments on the permeability of fatty acid films to water, whilst if in a more liquid condition it might have the opposite effect, since O_2 and CO are as a rule more soluble in oils than in water, and the diffusion constant, when measured in the units adopted above, is usually proportional to the solubility coefficient of the gas in the liquid. The possibility of such an increased diffusion constant has already been pointed out by Bayliss (1918).

The equation : volume of dissolved gas passing through the membrane per second

$$= (p - p') A \frac{D_m}{b_m}$$

cannot therefore, at present, be applied in the manner suggested above, but by applying it for determining a minimum value for the diffusion constant of unit area of the membrane, it can be shown that the diffusion through the membrane is rather unlikely to be an influence of much importance. Such a minimum value will also be of use later. It is obtained as follows :—

Consider the quickest of the four processes listed in Table I, viz., $O_2 + Hb \rightarrow O_2Hb$. The average p_{O_2} (= partial pressure of dissolved O_2) in the surrounding fluid whilst per cent. O_2Hb rises from 0 per cent. to 50 per cent. = 50 mm. Hg approximately.

Average initial p_{O_2} in corpuscle = 0 mm. Hg.

Rate of uptake of O_2 = 8 c.c. O_2 N.T.P. per second per cubic centimetre corpuscle contents approximately.

If it be assumed that the average pressure difference of 50 mm. Hg is *all* used in driving the dissolved O_2 through the corpuscle membrane, instead of only in part as must actually be the case, then the value of D_m/b_m as calculated by applying equation (I) to these data, will clearly be a minimum one.

Let n = number of corpuscles required to make up a volume of 1 c.c.,

$2b_c$ = average thickness of whole corpuscle (Ponder, 1929)

$$= 1.6 \times 10^{-4} \text{ cm.}$$

Then nA (area of corpuscle) $\times 2b_c = 1$ c.c.

Applying equation (I), with the assumptions that the corpuscle can be regarded as a flat disc with two faces, each of area A , and that diffusion through the rim can be neglected

$$50 \text{ mm. Hg} \times \text{minimum value of } \frac{D_m}{b_m} \times nA = 8 \text{ c.c. per second,}$$

whence

$$50 \times \text{minimum value of } \frac{D_m}{b_m} \times \frac{1}{b_c} = 8,$$

therefore

$$\begin{aligned}\text{minimum value of } \frac{D_m}{b_m} &= \frac{8 \times 1.6 \times 10^{-4}}{100} \\ &= 1.3 \times 10^{-5}.\end{aligned}$$

Now although, as already pointed out, the value of D_m and b_m are not known, it is of interest to see what would be the minimum value to be assigned to D_m if $b_m = 10^{-6}$ cm., *i.e.*, the maximum thickness suggested by the electrical measurements.

$$\text{Since minimum value of } \frac{D_m}{b_m} = 1.3 \times 10^{-5}$$

Minimum value of D_m in this case would $= 1.3 \times 10^{-11}$.

This is only one-fiftieth of the value of D_{water} for O_2 which $= 6.4 \times 10^{-10}$ in the present units. It would certainly be surprising if the value of D_m were suppressed as much as this. This consideration makes it rather unlikely that the influence of diffusion through the corpuscle membrane is large even in the case of the fastest process $O_2 + Hb \rightarrow O_2Hb$, and tends to justify the tentative procedure sketched out at the end of this paper, namely, that of working out the kinetics of the whole process on the assumption that only diffusion and chemical reaction within the corpuscle need be considered, and then of planning further experiments to check the formulæ arrived at on this hypothesis.

For the present, however, we shall take the minimum value of D_m/b_m as given *rigorously* by the above equation and see to what extent the other three processes, *viz.*, $CO + Hb \rightarrow COHb$, $O_2Hb \rightarrow O_2 + Hb$, $COHb + O_2 \rightarrow O_2Hb + CO$ would be affected by diffusion through the corpuscle membrane on this basis.

Influence of diffusion through corpuscle membrane on:—

(i) *The reaction* $CO + Hb \rightarrow COHb$.—According to Table I, this reaction proceeds 2.8 times more slowly than $O_2 + Hb \rightarrow O_2Hb$ in corpuscle. Assuming that

$$\frac{D_m \text{ for CO}}{D_m \text{ for } O_2} = \frac{D_{\text{water for CO}}}{D_{\text{water for } O_2}} = 0.89,$$

then the maximum extent to which the reaction would be impeded by diffusion through the corpuscle membrane

$$= \frac{100}{2.8 \times 0.89} = 40 \text{ per cent.}$$

(ii) *The reaction* $O_2Hb \rightarrow O_2 + Hb$.—According to Table I, the time taken for the per cent. O_2Hb to fall to 50 per cent. $= 0.4$ seconds.

Assuming that the gas combining capacity of 1 c.c. of corpuscle contents = 0.4 c.c.

Maximum value of $p - p'$ is given by

$$(p - p') \times \text{minimum value of } \frac{D_m}{b_m} \times \frac{2nAb_c}{b_c} = \frac{0.4 \times 50 \text{ per cent.}}{0.4} = 0.5 \text{ c.c. per second,}$$

or

$$\text{maximum value of } (p - p') = \frac{0.5 \times 1.6 \times 10^{-4}}{2 \times 1.3 \times 10^{-5}} = 3 \text{ mm. Hg.}$$

At any moment the p_{O_2} within the corpuscle \leq the value which would be in equilibrium with the per cent. O_2Hb at that moment.

From the average dissociation curve of sheep's blood at p_H 7.4, temperature 15° C. it can be shown that—

at 90 per cent. O_2Hb p_{O_2} in equilibrium = about 6 mm.

at 50 per cent. O_2Hb p_{O_2} in equilibrium = about 3 mm.

Since the maximum value of $(p - p')$ is of the same magnitude as these pressures, the argument fails to exclude the possible influence of diffusion through the membrane upon this reaction.

(iii) *The reaction* $O_2 + COHb \rightarrow CO + O_2Hb$.—According to Table I, the time taken for the per cent. O_2Hb to fall to 50 per cent. = 60 seconds.

The maximum value of $p - p'$ by a similar calculation to that just given therefore = 0.022 mm. Hg CO.

At 50 per cent. $COHb$ —50 per cent. O_2Hb within the corpuscle, the equilibrium p_{CO} , when $p_{O_2} = 760$ mm. Hg = 3 mm.

Since this is 140 times greater than the maximum pressure difference of CO required across the corpuscle membrane, it follows that in this reaction the influence of the corpuscle membrane is certainly negligible.

The conclusion so far is therefore as follows. The influence of diffusion across the corpuscle membrane might be large (up to 40 per cent.) in the case of $CO + Hb \rightarrow COHb$, might be dominant in the case of $O_2Hb \rightarrow O_2 + Hb$, but is certainly negligible in the case of $O_2 + COHb \rightarrow CO + O_2Hb$.

(C) *Diffusion Through the Interior of the Corpuscle.*

Preliminary information as to the importance of this factor in the four processes listed in Table I may be obtained by the application of Fourier diffusion equations.

Consider a plane sheet of liquid of thickness $2b$.

Let the liquid be exposed on either side to dissolved gas at pressure p_b .
 Let the partial pressure of dissolved gas at time $t = 0$ in the liquid be p_0 .
 Then the equation for the diffusion of gas into the sheet is

$$D \frac{\partial^2 p}{\partial x^2} = a \frac{\partial p}{\partial t},$$

where

D = diffusion constant of the gas in the liquid (in the units given on p. 5).

a = solubility coefficient of the gas in the liquid (in the units given on p. 5).

x = distance from the centre of the sheet in centimetres. (II)

At time t , the average partial pressure of dissolved gas within the sheet is given by

$$\bar{p} = p_b \left[1 - \frac{p_b - p_0}{p_b} \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} e^{-\frac{D}{a} \left(\frac{2n+1}{2b} \pi \right)^2 t} \right]. \quad (\text{III})$$

The general validity of this equation was in effect verified by Loschmidt (1870) in his classical experiments on the interdiffusion of gases. It has been used for physiological purposes by Hill (1928) in connection with the supply of O_2 to muscle and nerve, and by Roughton (1925) in connection with gas distribution within the capillary.

Practical Use of Equation (III).

(i) If

$$\frac{D}{a} \frac{\pi^2}{4b^2} t > 0.4, \text{ all terms in } \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} e^{-\frac{D}{a} \left(\frac{2n+1}{2b} \pi \right)^2 t}$$

can be neglected in comparison with the first, and (III) reduces to

$$\bar{p} = p_b \left[1 - \frac{p_b - p_0}{p_b} \cdot \frac{8}{\pi^2} e^{-\frac{D}{a} \frac{\pi^2}{4b^2} t} \right]$$

to within 0.4 per cent., whence

$$\frac{\bar{p} - p_0}{p_b - p_0} = 1 - \frac{8}{\pi^2} e^{-\frac{D}{a} \frac{\pi^2}{4b^2} t}. \quad (\text{III A})$$

(ii) If

$$0 < \frac{D}{a} \frac{\pi^2}{4b^2} t < 0.4.$$

Ingham (1932)* has shown that

$$1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} e^{-\frac{D}{a} \left(\frac{2n+1}{2b} \pi \right)^2 t} = \frac{2}{b} \sqrt{\frac{Dt}{a\pi}}$$

* *Vide* also Roseburgh and Miller (1910).

to within < 0.2 per cent., therefore

$$\bar{p} = p_b \left[1 - \frac{p_b - p_0}{p_b} \left(1 - \frac{2}{b} \sqrt{\frac{Dt}{a\pi}} \right) \right] \quad (\text{III B})$$

whence

$$\frac{\bar{p} - p_0}{p_b - p_0} = \frac{2}{b} \sqrt{\frac{Dt}{a\pi}},$$

since $\frac{D}{a} \frac{\pi^2}{b^2} t$ must always be positive.

Any actual case can therefore be treated simply and quickly by means of (III A) or (III B).

Application of equations (III A), (III B), to the reactions in the corpuscle.

Let a_{O_2} = solubility coefficient of O_2 within corpuscle

a_{CO} = solubility coefficient of CO within corpuscle

D_{O_2} = diffusion constant of O_2 within corpuscle

D_{CO} = diffusion constant of CO within corpuscle

$2b$ = average thickness of corpuscle.

Numeral Values.—Van Slyke and Sendroy (1928) have shown that for hydrogen a (in corpuscle) = 90 per cent. of a for water.

Assuming that the same ratio holds good for O_2 and CO :

$a_{O_2} = 0.031$ c.c. per atmosphere = 4.1×10^{-5} c.c. O_2 per cubic centimetre corpuscle contents per millimetre Hg.

$a_{CO} = 0.023$ c.c. per atmosphere = 3×10^{-5} c.c. O_2 per cubic centimetre corpuscle contents per millimetre Hg.

There are no data for the diffusion constants of O_2 and CO within the corpuscle, but Krogh (1919) has shown that the diffusion constant of O_2 through a 20 per cent. gelatine gel = about 80 per cent. of D_{O_2} for water. It seems reasonable to suppose that the value for the interior of the corpuscle is not far different.

On this basis we take

$D_{O_2} = 5 \times 10^{-10}$ c.c. O_2 per second per square centimetre per 1 mm. Hg per centimetre.

$D_{CO} = 3.7 \times 10^{-10}$.

The thickness of the sheep's corpuscle according to Ponder (1929) varies from 1.4μ at the centre to 1.7μ at the periphery. According to Hartridge

(1920) and Ponder (1925), the biconcave shape of the corpuscle with the rim at the periphery tends to ensure that diffusion will take place at the same rate in all parts of the corpuscle. In the experiments in the reaction velocity apparatus the fluid around the corpuscles was found to be kept so well stirred that it is permissible to regard the whole surface of the corpuscle as an equipotential surface, the oxygen concentration of which is practically equal to the average oxygen concentration in the surrounding fluid. Starting from this surface the lines of flow into the interior would tend to be convergent at the thickened edges of the corpuscle, and to be parallel near the thinner centre. Rough calculations by the author confirm that, in the case where the diffusing substance does not engage in chemical reaction, the average rate of saturation at the edge would be about the same (to within perhaps 20 per cent.) as in the centre, the greater thickness of the edge being compensated for by the convergence of the lines of flow. It has therefore seemed legitimate, for purposes of simplification, to assume that for the calculations which follow, the corpuscle can be replaced by a flat disc of thickness equal to the minimum thickness at the centre of the actual corpuscle, and to neglect the effect of the rim.

Hence $2b$ is assumed $= 1.4 \times 10^{-4}$ cm.

With the above values of a , D , $2b$, the following table of values is obtained for the speed with which diffusion equilibrium would be approached.

Table II.

t in seconds.	$\frac{\bar{p} - p_0}{p_b - p_0}$ for O_2 or CO .
0.00005	0.12
0.00001	0.18
0.0001	0.554
0.0002	0.755
0.0003	0.866
0.0004	0.928
0.0005	0.960
0.0006	0.978
0.0007	0.988
0.0008	0.993
0.0009	0.996
0.0010	0.998

An idea as to how far diffusion within the corpuscle has a controlling effect upon the various processes of Table I can be got by considering the chemical reaction, and the diffusion within the corpuscle as occurring in successive stages instead of as going hand in hand, as in fact they must. This method will be more fully understood when the various cases are considered.

(i) *Rate of* $O_2 + Hb \rightarrow O_2Hb$.—Let the corpuscle be initially free from O_2 , but exposed to a partial pressure $p_b = 75$ mm. Hg. in the surrounding fluid.

From Table II the amount of O_2 which would dissolve in the corpuscle if there was no chemical reaction therein would be 0.0028 c.c. O_2 per cubic centimetre corpuscle in 0.0004 second. And

$$\bar{p}/p_b = 92.8 \text{ per cent.},$$

therefore

$$\bar{p} = 92.8 \text{ per cent. of } 75 \text{ mm.} = 70 \text{ mm. Hg.}$$

Now if the chemical reaction within the corpuscle was so slow that the amount of O_2 used by it in the 0.0004 second was small in comparison with 0.0028 c.c. then clearly the influence of diffusion within the corpuscle would be small. *Vice versa* if the contrary is found to be the case.

Supposing p_{O_2} in corpuscle = 70 mm. Hg, how much O_2Hb would actually be formed by the chemical reaction in 0.0004 second?

Assuming the kinetics of the process is the same as in solution, the answer from Table I is 18.7 per cent. O_2Hb .

With a gas combining capacity of 0.4 c.c. O_2 per cubic centimetre corpuscle, this equals 0.075 c.c. O_2 per cubic centimetre corpuscle.

Since this is 25 times greater than the amount (0.0028 c.c. O_2) which could dissolve in the time if there was no chemical reaction, it is clear that diffusion within the corpuscle *must* be a pronounced limiting factor in the present process.

(ii) *Rate of* $CO + Hb \rightarrow COHb$.—The velocity constant of the chemical reaction in this case is 10 times smaller than in the case of $O_2 + Hb$.

The corresponding figures for a time interval of 0.0004 second are 0.0021 c.c. CO (instead of 0.0028 c.c. O_2) in physical solution, and for the chemical reaction 0.0075 c.c. CO (instead of 0.075 c.c. O_2).

Since the latter figure is 3.6 times larger than the former, the influence of diffusion within the corpuscle must also in this case be significant though less serious than in the case of $O_2 + Hb$.

In the next two cases the argument is slightly different, but does not need any special preliminary explanation.

(iii) *Rate of* $O_2Hb \rightarrow O_2 + Hb$.—Consider the following case.

Let the per cent. of O_2Hb in the corpuscle be 90.

The p_{O_2} which would be in equilibrium therewith at p_H 7.4, temperature 15° C. = about 6 mm.

In 0.0004 second the chemical reaction if unimpeded would cause the per cent. of O_2Hb (by Table I) to fall by 0.36.

The O_2 therefrom if liberated into physical solution in the corpuscle would cause a p_{O_2} of 36 mm. Hg.

In 0.0004 second diffusion could reduce this pressure by 92.8 per cent. Table II), *i.e.*, to 2.6 mm. Hg.

Since this is only 40 per cent. of the equilibrium p_{O_2} (*i.e.*, 6 mm.) at 90 per cent. O_2Hb , it seems probable that the influence of diffusion within the corpuscle on this process is relatively small, though still appreciable.

(iv) *Rate of $O_2 + COHb \rightarrow CO + O_2Hb$.*—At 90 per cent. $COHb$, p_{CO} in equilibrium therewith = 30 mm. Hg about (if $p_{O_2} = 760$ mm. Hg).

In 0.0004 second the per cent. of $COHb$ would (by Table I) fall by 4.3×10^{-4} per cent. $COHb$ due to the unimpeded chemical reaction.

This amount of CO if liberated into physical solution in the corpuscle would cause a p_{CO} of 0.056 mm. Hg.

In 0.0004 second diffusion could reduce this to 0.004 mm. Hg, *i.e.*, to a value only 1/7500th that of the equilibrium p_{CO} .

If 50 per cent. $COHb$ is taken instead of 90 per cent. the figures obtained are 3 mm. and 0.002 mm. Hg respectively. Thus it may be safely concluded in the case of this process that the influence of diffusion within the corpuscle is quite negligible.

The conclusions so far arrived at may now be tabulated in the following rough form.

Table III.

Process in corpuscle.	Influence of diffusion through corpuscle membrane.	Influence of diffusion within interior of corpuscle.
$O_2 + Hb \rightarrow O_2Hb$	Not known	Very large
$CO + Hb \rightarrow COHb$	< 40 per cent.	Large
$O_2Hb \rightarrow O_2 + Hb$	< about 70 per cent.	< 50 per cent.
$O_2 + COHb \rightarrow CO + O_2Hb$	Negligible	Negligible.

(D) *The Kinetics of the Chemical Reactions within the Corpuscle.*

In the case of the reaction $O_2 + COHb \rightarrow CO + O_2Hb$, it has now been shown that the two factors, other than that of the true chemical reaction velocity itself, which might restrict the rate of process within the corpuscle, must have a negligible limiting influence. Since, according to Table I, the observed rate of this process is the same in the corpuscle as in solution, it seems fair to conclude that the chemical kinetics of the process must be practically the same in the corpuscle as in solution, in spite of the quite different

conditions—especially that of hæmoglobin concentration—prevailing in the corpuscle. There does not seem to be any *a priori* reason why, if the chemical kinetics of this process are the same as in solution, the same should not apply to the other processes likewise, and it therefore seems justifiable to extend the analysis further on this basis, as will be done in a preliminary manner in the remainder of this paper.

SECTION II.

Equations for the Processes within the Corpuscle.

The discussion in Section I, subsection B, as to the function of the corpuscle membrane was inconclusive, though from the calculations there given, and also on teleological grounds, it would seem unlikely that the membrane acts as an important limiting factor. The writer has therefore sought to calculate the rate of uptake of O_2 and of CO by a layer of reduced hæmoglobin of the same concentration and equivalent thickness as in the corpuscle. This procedure has not only enabled him to get a far better idea of the action of diffusion within the corpuscle than that given by the methods of Section I, but also has suggested a large number of further interesting experiments. Furthermore, as has been mentioned in the Introduction, processes in which chemical reaction, velocity and diffusion rate are jointly limiting factors must be very common in physiology, but have not, so far as the writer is aware, been previously worked out by physical chemists. For these reasons it seemed worth while to place on record a rather detailed account of the theoretical investigation of a process and system of this composite type. The remainder of this paper is devoted to description of the details of this undertaking, namely the assumption involved, the mode of formulating and of attempting to solve the necessary theoretical equations, and the conclusions to be drawn from the comparison between theory and experiment, together with a sketch of the further experiments suggested by the theoretical results.

A. *Basal Assumptions.*

In calculating the rate of uptake of O_2 or CO by a layer of reduced hæmoglobin of the same equivalent thickness as that of the corpuscles, the following assumptions will be made :—

(i) That the velocity of the reaction in the corpuscle follows the same course as in Hb solution, which is empirically (Hartridge and Roughton, 1925) equal to

$$k' (O_2) (Hb) - k (O_2Hb), \quad (IV)$$

where k , k' are the velocity constants of the reaction, (O_2) is the concentration of dissolved O_2 , (Hb) is the concentration of hæmoglobin, and (O_2Hb) is the concentration of oxyhæmoglobin expressed in equivalents (equivalent weight = 17000) per unit volume.

Some justification for this assumption has been given in Section I, (D).

In the present treatment the initial part of the process will only be considered, so that the back reaction term $k(O_2Hb)$ can be neglected.

(ii) That the *surface* of the corpuscle is exposed to a constant pressure of dissolved gas throughout the process under investigation.

It will be remembered that the experiments quoted in the Introduction showed that the stirring was so thorough that there were no appreciable gradients of dissolved gas in the fluid surrounding the corpuscles during the actual uptake of dissolved gas by the corpuscles in the rapid reaction apparatus. The partial pressure of dissolved gas *immediately adjacent* to the corpuscles must therefore have been, at each moment, practically the same as the average partial pressure throughout the fluid between the corpuscles. It is just this circumstance which makes it possible to apply Fourier analysis to the present problem, and which indeed differentiates it from somewhat analogous problems in conduction of heat* wherein physicists have been forced to abandon Fourier analysis and to fall back on purely experimental work assisted by the "method of dimensions." In order that assumption (ii) should be in harmony with experimental conditions, it is also necessary that the total amount of gas in the fluid around the corpuscles should be so large that the average partial pressure does not fall appreciably during the process.

(iii) That the hæmoglobin is uniformly distributed throughout the thickness of the corpuscle, and hence that a layer of concentrated hæmoglobin can be regarded as "equivalent" to the corpuscle in the sense used in this section.

This view is fairly generally accepted. Even if the hæmoglobin in the corpuscle was present in the solid condition, it would still have to occupy more than one-quarter of the total thickness of the corpuscle, for the density of solid hæmoglobin is about 1.3, and the weight of hæmoglobin present in each cubic centimetre of corpuscle contents is about 0.3 to 0.4 gm.

(iv) That the diffusion of hæmoglobin molecules within the corpuscle is so slow in comparison with the diffusion of O_2 molecules that the hæmoglobin can be regarded as stationary in the corpuscle, and hence also in the mathematical model used here. This must obviously be true if there is any "struc-

* Such as the transference of heat from rapidly streaming fluids to the walls of solid tubes.

ture," gel or otherwise, within the corpuscle. For, one of the original distinctions drawn by Thomas Graham between crystalloids such as dissolved O_2 and colloids such as hæmoglobin rested on the observation that the rate of diffusion of the former was only slightly affected even by dense "structure," whereas the rate of diffusion of the latter was most readily reduced, if not obliterated, even by the sparse structure existing in very dilute gels. This observation has been amply confirmed by subsequent workers. Physiologists, however, are not at present agreed as to whether the interior of the corpuscle contains any structure or whether, on the other hand, it consists merely of an extremely concentrated solution of proteins. Even in the latter eventuality, the influence of diffusion of the hæmoglobin must become relatively smaller and smaller, as the p_{O_2} in the surrounding fluid is increased, so that if the p_{O_2} is larger than a limiting value the diffusion of Hb will become relatively negligible in the theoretical calculation. As to the magnitude of this lower limit, no precise information can be obtained without a knowledge of the diffusion constant of Hb in 30 per cent. solution. Although experimental data are only available for Hb solutions of 1 to 2 per cent., it is possible, as shown in the Appendix to this paper, to obtain a probable upper limit to the diffusion constant in 30 per cent. solution. On this basis it is found (*vide* Appendix) that if p_{O_2} or p_{CO} in the surrounding fluid equals 75 mm. Hg., the influence of Hb diffusion as compared with O_2 or CO diffusion is less than 30 per cent whereas if p_{O_2} or p_{OC} equals 750 mm. Hg, the Hb diffusion is relatively less than 10 per cent. in importance. For the approximate purposes of this paper, it has therefore been feasible to neglect the diffusion of Hb. Had this not been the case, the equations would probably have proved insoluble.

(v) For the purposes of this calculation the corpuscle has again been regarded as equivalent to an infinite flat sheet of thickness equal to the minimum thickness of the actual corpuscle.

Some justification for this has already been given, in Section I, (C), for the case of diffusion unaccompanied by chemical reaction. But for combined diffusion and chemical reaction velocity, rough calculations like those referred to on p. 14 have unfortunately been so far impossible. But since in the present instance the shape of the lines of flow must be generally the same as in the preceding simpler case, it does not seem to the writer too rash to assume that approximately the same conclusion can hold good.

Finally, it may be emphasised that, even if future work were to show that these basal assumptions had been too drastic in their simplicity (and had therefore led to erroneous results when applied to the rapid reaction corpuscle

experiments), the treatment below should still be of interest to physiologists as an example of the study of the influence of combined diffusion and chemical reaction velocity in an ideal system, which is not so very far removed from conditions prevailing in the physical chemistry of the cell.

B. *Maximum Rate and Minimum Rate Solutions for Rate of Uptake of*
O₂ (or CO).

With the above assumptions the equation for the rate of uptake of O₂ by the layer of hæmoglobin is given by the equation

$$D \frac{\partial^2 p}{\partial x^2} = a \frac{\partial p}{\partial t} + k'py, \quad (V)$$

where p is the partial pressure of dissolved O₂ at time t at a distance x from the centre of the layer, y the concentration of reduced Hb expressed in equivalents at this point and instant of time, a the solubility coefficient of oxygen in the layer (*vide* p. 5), and D the diffusion constant of O₂ in the layer (*vide* p. 4), $2b$ is equal to the thickness of layer.

The boundary conditions are as follows :--

- (1) At $t = 0$, $p = 0$ for all values of x .
- (2) At $x = b$, $p = p_b$ for all values of t .
- (3) At $x = 0$, $\partial p / \partial x = 0$ for all values of t .

We shall first proceed to solve this equation for the earliest stages of the process during which y may be taken as practically constant equal to y_0 (initial concentration of reduced Hb).

The equation (I) may be then written

$$D \frac{\partial^2 p}{\partial x^2} = a \frac{\partial p}{\partial t} + \kappa p,$$

where $\kappa = k'y_0$.

The solving of this equation follows the standard methods adopted in the theory of conduction of heat (the term κp , however, corresponding to a radiation of heat from the surface of the solid through which the heat is passing).

The solution so obtained is as follows :--

$$p = p_b \left[\frac{\cosh \left(\sqrt{\frac{\kappa}{D}} x \right)}{\cosh \left(\sqrt{\frac{\kappa}{D}} b \right)} - \frac{2}{b} \sum_{n=0}^{\infty} \frac{(-1)^n \frac{2n+1}{2b} \pi}{\left(\frac{2n+1}{2b} \pi \right)^2 + \frac{\kappa}{D}} e^{-\left(\frac{2n+1}{2b} \pi \right)^2 \frac{D}{a} t - \frac{\kappa}{a} t} \cos \frac{2n+1}{2b} \pi x \right] \quad (VI)$$

N.B.—When $\kappa = 0$, i.e., there is no chemical reaction, this reduces to the well-known form

$$p = p_b \left[1 - \frac{4}{\pi} \sum_0^{\infty} \frac{1}{2n+1} e^{-\left(\frac{2n+1}{2b}\pi\right)^2 \frac{D}{a} t} \cos \frac{2n+1}{2b} \pi x \right].$$

In physical solution at time t the volume of O_2 per cubic centimetre

$$\begin{aligned} &= \frac{a}{b} \int_0^b p \, dx = \frac{a}{b} p_b \left[\sqrt{\frac{D}{\kappa}} \tanh \left(\sqrt{\frac{\kappa}{D}} b \right) \right. \\ &\quad \left. - \frac{2}{b} \sum_0^{\infty} \frac{1}{\left(\frac{2n+1}{2b}\pi\right)^2 + \frac{\kappa}{D}} e^{-\left(\frac{2n+1}{2b}\pi\right)^2 \frac{D}{a} t - \frac{\kappa}{a} t} \right]. \quad (\text{VII}) \end{aligned}$$

Note when $\kappa = 0$ this reduces to the well-known form

$$ap_b \left[1 - \frac{8}{\pi^2} \sum_0^{\infty} \frac{1}{(2n+1)^2} e^{-\left(\frac{2n+1}{2b}\pi\right)^2 \frac{D}{a} t} \right]. \quad (\text{VIII})$$

In chemical combination at time t the volume of O_2 per cubic centimetre

$$\begin{aligned} &= \frac{\kappa}{b} \int_0^t \int_0^b p \, dt \, dx \\ &= \frac{\kappa}{b} p_b \left[t \sqrt{\frac{D}{\kappa}} \tanh \left(\sqrt{\frac{\kappa}{D}} b \right) \right. \\ &\quad \left. - \frac{2}{b} \sum_0^{\infty} \frac{1 - e^{-\left(\frac{2n+1}{2b}\pi\right)^2 \frac{D}{a} t - \frac{\kappa}{a} t}}{\left(\left[\frac{2n+1}{2b}\pi\right]^2 + \frac{\kappa}{D}\right)\left(\left[\frac{2n+1}{2b}\pi\right]^2 \frac{D}{a} + \frac{\kappa}{a}\right)} \right]. \quad (\text{IX}) \end{aligned}$$

This "initial rate" solution, unfortunately, gives but little guide as to the subsequent course of the reaction, since it is often found that diffusion processes of this type start at a very high rate and then soon drop to more moderate rates. Such, for example, is the case for diffusion, unaccompanied by chemical reaction, for which an exact solution over the whole time range is obtainable as in equation (III) and Table II of this paper.

The writer has been unable to get a general solution of the equation

$$D \frac{\partial^2 p}{\partial x^2} = a \frac{\partial p}{\partial t} + k' p y,$$

but he has been able to obtain two further equations, the first of which gives a value always *greater*, and the second a value always *less* than the true rate. The true theoretical rate must therefore lie between this maximum and minimum value. Later it will be shown that the maximum and minimum rates lie close enough together to fix the true rate to within ± 10 per cent. over a large range for each process.

The maximum rate solution is obtained as follows. For all finite values of t every term in

$$\sum_0^{\infty} \frac{1 - e^{-\left(\frac{2n+1}{2b}\pi\right)^2 \frac{D}{a} t - \frac{\kappa}{a} t}}{\left[\left(\frac{2n+1}{2b}\pi\right)^2 + \frac{\kappa}{D}\right] \left[\left(\frac{2n+1}{2b}\pi\right)^2 + \frac{\kappa}{a}\right]}$$

is positive, therefore from equation (IX), the uptake in time t

$$\left. \begin{aligned} &< \frac{p_b t}{b} \sqrt{\kappa D} \tanh \left(\sqrt{\frac{\kappa}{D}} \cdot b \right) \\ &< \frac{p_b t}{b} \sqrt{k' y_0 D} \tanh \left(\sqrt{\frac{k' y_0}{D}} \cdot b \right) \end{aligned} \right\} \quad (\text{IXA})$$

N.B.—When $b > 0$, this expression $< p_b k' y_0 t$, but when $b \rightarrow 0$, i.e., when the influence of diffusion becomes negligible as compared with the chemical reaction velocity, then

$$\frac{p_b t}{b} \sqrt{k' y_0 D} \tanh \left(\sqrt{\frac{k' y_0}{D}} b \right) \rightarrow p_b k' y_0 t,$$

which is physically correct, since $p_b k' y_0 t$ would be the uptake of gas in time t due to the chemical reaction alone, if the layer was uniformly saturated with gas at constant partial pressure p_b during the interval $0 \rightarrow t$.

Let \bar{y} = average value of y in the layer at time t . Then

$$-\frac{d\bar{y}}{dt} < \frac{p_b}{b} \sqrt{k' \bar{y} D} \tanh \left(\sqrt{\frac{k' \bar{y}}{D}} \cdot b \right), \quad (\text{IXB})$$

for this last expression would be greater than the rate at time t , if all the gas which had entered before t were distributed uniformly within the layer at time t (instead of, as in actual fact, being distributed in a gradient), whereas the true rate at time t must be less than this latter rate.

Integrating

$$\sqrt{y_0} - \sqrt{\bar{y}} < \frac{1}{2} \frac{p_b t}{b} \sqrt{k' D} \tanh \left(\sqrt{\frac{k' y_0}{D}} \cdot b \right), \quad (\text{IXC})$$

The "minimum rate" solution can be obtained in two different forms, the first of which has been found to be more valuable in the cases treated below, though in other instances the second solution is to be preferred.

I. Under the physical conditions of this problem in the equation

$$D \frac{\partial^2 p}{\partial x^2} = a \frac{\partial p}{\partial t} + k' p y,$$

$\partial p / \partial t$ is always positive, and a is positive, therefore

$$D \frac{\partial^2 p}{\partial x^2} > k' p y.$$

Now $p_{atx,t}$ must be $> p_{atx,0}$.

Also $y_{atx,t}$ must be $> y_{atb,t}$.

Since

$$\left(\frac{dy}{dt} \right)_{x=b} = -k' p_b y,$$

therefore

$$\log \left(\frac{y}{y_0} \right)_{atx=b} = -k' p_b t,$$

therefore

$$y_{atb,t} = y_0 e^{-k' p_b t},$$

therefore

$$D \frac{\partial^2 p}{\partial x^2} > k' p y_0 e^{-k' p_b t} > \lambda p,$$

where $\lambda = k' y_0 e^{-k' p_b t}$, therefore

$$D \frac{\partial^2 p}{\partial x^2} \cdot \frac{\partial p}{\partial x} > \lambda p \frac{\partial p}{\partial x},$$

therefore

$$D \frac{\partial}{\partial x} \left[\left(\frac{\partial p}{\partial x} \right)^2 \right] > \lambda \frac{\partial}{\partial x} [p^2],$$

therefore

$$\left[D \left(\frac{\partial p}{\partial x} \right)^2 \right]_0^b > [\lambda p^2]_0^b.$$

Since $\left(\frac{\partial p}{\partial x} \right)_0 = 0$, therefore

$$D \left(\frac{\partial p}{\partial x} \right)_b^2 > \lambda (p_b^2 - p_0^2),$$

therefore

$$\left(\frac{\partial p}{\partial x} \right)_b > p_b \sqrt{\frac{\lambda}{D}} \cdot \sqrt{1 - \frac{p_0^2}{p_b^2}},$$

therefore

$$\begin{aligned} \int_0^t \frac{D}{b} \left(\frac{\partial p}{\partial x} \right)_b dt &> \frac{D p_b}{b} \int_0^t \sqrt{\frac{\lambda}{D}} \cdot \sqrt{1 - \frac{p_0^2}{p_b^2}} dt \\ &> \frac{p_b}{b} \sqrt{D k' y_0} \int_0^t e^{-\frac{k' p_b t}{2}} \sqrt{1 - \frac{p_0^2}{p_b^2}} dt. \end{aligned}$$

For very small values of t , it follows from (IX) that

$$p_0 < \frac{p_b}{\cosh \left(\sqrt{\frac{k' y_0}{D}} b \right)}.$$

To obtain a similar inequality over the whole time interval $0 - t$, $k' y_0$ in the latter equation must be replaced by $k' y_0 e^{-k' p_b t}$.

The reason for this is as follows. From the physics of the problem it can be seen that the smaller the value of $k'y$ in the equation

$$D \frac{\partial^2 p}{\partial x^2} = a \frac{\partial p}{\partial t} + k' p y,$$

the greater will be the extent to which the gas arriving at any particular cross section of the corpuscle will increase the amount of gas in physical solution (and hence increases p) at that point rather than increase the amount in chemical combination, and therefore the larger will be the value of p_0/p_b at time t . Now the expression $k' y_0 e^{-k' p_b t}$ must be less than $k'y$ over the whole interval $0 - t$, and therefore

$$p_0 < \frac{p_b}{\cosh \left[\sqrt{\frac{k' y_0}{D}} \cdot b \cdot e^{-\frac{k' p_b t}{2}} \right]},$$

over the interval $0 - t$.

Now

$$\int_0^t \frac{D}{b} \left(\frac{\partial p}{\partial x} \right)_b dt = \frac{\text{total uptake of gas by the layer in time } t}{\text{volume of layer}},$$

therefore total uptake per cubic centimetre

$$\begin{aligned} &> \frac{p_b}{b} \sqrt{k' y_0 D} \int_0^t e^{-\frac{k' p_b t}{2}} \left[1 - \frac{1}{\cosh^2 \left(b \sqrt{\frac{k' y_0}{D}} e^{-\frac{k' p_b t}{2}} \right)} \right] dt \\ &> -\frac{2}{b} \sqrt{\frac{D y_0}{k'}} \int_0^t \tanh \left(b \sqrt{\frac{k' y_0}{D}} z \right) dz, \quad \text{where } z = e^{-\frac{k' p_b t}{2}} \\ &> \frac{2}{b^2} \frac{D}{k'} \log_e \left\{ \frac{\cosh \left(\sqrt{\frac{k' y_0}{D}} \cdot b \right)}{\cosh \left(\sqrt{\frac{k' y_0}{D}} \cdot b \cdot e^{-\frac{k' p_b t}{2}} \right)} \right\}. \end{aligned}$$

Now total uptake of gas per cubic centimetre equals uptake of gas in chemical combination per cubic centimetre plus uptake of gas in physical solution per cubic centimetre.

The uptake of gas in physical solution per cubic centimetre is less than ap_b since ap_b is the final equilibrium value of gas in physical solution as $t \rightarrow \infty$.

Therefore uptake of gas in chemical combination per cubic centimetre

$$> \frac{2}{b^2} \frac{D}{k'} \log_e \left\{ \frac{\cosh \left(b \sqrt{\frac{k'y_0}{D}} \right)}{\cosh \left(b \sqrt{\frac{k'y_0}{D}} e^{-\frac{k'p_b t}{2}} \right)} \right\} - ap_b \quad (X)$$

II. The second minimum rate solution is also obtained by a combination of mathematical and physical reasoning.

For all values of x and t , $y \geq y_0 e^{-k'p_b t}$ (i.e., the value of y at the periphery at time t , *vide* p. 22).

For very small values of t

$$p = p_b \left[\frac{\cosh \left(\sqrt{\frac{k'y_0}{D}} x \right)}{\cosh \left(\sqrt{\frac{k'y_0}{D}} b \right)} - \frac{2}{b} \sum_0^\infty \frac{(-1)^n \frac{2n+1}{2b} \pi}{\left(\frac{2n+1}{2b} \pi \right)^2 + \frac{K}{D}} e^{-\left(\frac{2n+1}{2b} \pi \right)^2 \frac{D}{a} t - \frac{K}{a} t} \cos \frac{2n+1}{2b} \pi x \right]$$

In general by the same type of argument as on p. 24, p will be greater than the expression just given.

Therefore gas uptake in chemical combination in time t per unit volume

$$> \frac{k'p_b y_0}{b} \int_0^t \int_0^b \left[\frac{e^{-k'p_b t} \cosh \left(\sqrt{\frac{k'y_0}{D}} x \right)}{\cosh \left(\sqrt{\frac{k'y_0}{D}} b \right)} - \frac{2}{b} \sum_0^\infty \frac{(-1)^n \frac{2n+1}{2b} \pi}{\left(\frac{2n+1}{2b} \pi \right)^2 + \frac{K}{D}} \cdot e^{-\left(\frac{2n+1}{2b} \pi \right)^2 \frac{D}{a} t - \frac{K}{a} t - k'p_b t} \cos \frac{2n+1}{2b} \pi x \right] dt dx$$

$$> \frac{1}{b} \sqrt{\frac{Dy_0}{k'}} \left[1 - e^{-k'p_b t} \right] \tanh \left(\sqrt{\frac{k'y_0}{D}} \cdot b \right) - \frac{2k'y_0 p_b}{b^2} \sum_0^\infty \frac{1 - e^{-\left(\frac{2n+1}{2b} \pi \right)^2 \frac{D}{a} t - \frac{K}{a} t - k'p_b t}}{\left[\left(\frac{2n+1}{2b} \pi \right)^2 + \frac{k'y_0}{D} \right] \left[\left(\frac{2n+1}{2b} \pi \right)^2 \frac{D}{a} + \frac{k'y_0}{a} + k'p_b \right]} \quad (X_A)$$

Numerical and Experimental Results.

C. In order to deduce numerical results from the equations developed in the previous subsection, it is necessary to assign values to the various constants in the equations.

Of these $2b$, a_{O_2} , a_{CO} , D_{O_2} , D_{CO} have already been dealt with in Section I, and the same values will be assumed.

In addition, the following assumption will be made :-

(i) y_0 = the initial concentration of reduced haemoglobin in the corpuscle.

This will be expressed in cubic centimetres of oxygen-combining capacity per cubic centimetre of corpuscle contents, and the actual value will be taken as 0.4 c.c. per cubic centimetre.

(ii) p_b = the constant pressure of dissolved gas at the edge of the corpuscle.

This will be taken as 75 mm. Hg, the usual value at $t = 0$ in most of the experiments in Hartridge and Roughton's paper (1927).

(iii) k' = the velocity constant of the reaction $O_2 + Hb \rightarrow O_2Hb = 3.0$, i.e.,

$$-\frac{dy \text{ (in same units as } y_0)}{dt \text{ (in seconds)}} = 3.3 \times p_{O_2} \text{ (in millimetres Hg)} \times y.$$

(iv) l' = the velocity constant of the reaction $CO + Hb \rightarrow COHb = 0.33$ (average of Roughton's unpublished experiments), i.e.,

$$-\frac{dy}{dt} = 0.33 \times p_{CO} \times y.$$

(I) For O_2 .

The results are given in Table IV and plotted in fig. 1. In this table the first column shows the time in seconds, and the second column the maximum rate of uptake of O_2 as given by the equation

$$\sqrt{y_0} - \sqrt{y} < \frac{1}{2} \frac{p_b t}{b} \sqrt{k'D} \tanh \left(\sqrt{\frac{k'y_0}{D}} \cdot b \right);$$

or inserting the numerical values $p_b = 75$, $b = 7 \times 10^{-5}$, $k' = 3.3$, $D = 5 \times 10^{-10}$

$$\sqrt{y_0} - \sqrt{y} < 21.9t.$$

The third column shows the minimum rate of uptake of O_2 as given by solution I, viz.,

$$y_0 - y > \frac{2}{b^2} \frac{D}{k'} \log_e \left\{ \frac{\cosh \left(\sqrt{\frac{k'y_0}{D}} \cdot b \right)}{\cosh \left(\sqrt{\frac{k'y_0}{D}} \cdot b e^{-\frac{k'p_b t}{2}} \right)} \right\} - ap_b$$

$$> 6.12 \times 10^{-2} \times \log_e \left\{ \frac{\cosh 3.6}{\cosh (3.6 \times e^{-125t})} \right\} - 3.07 \times 10^{-3}.$$

The fourth column shows the rate of the reaction in solution as calculated from the equation

$$\frac{dy}{dt} = -k'p_by = -250y.$$

Table IV.— O_2 uptake, expressed in per cent. Oxyhæmoglobin.

<i>t.</i>	Maximum rate.	Minimum rate I.	Rate in solution.
	per cent.	per cent.	per cent.
0.000	0	0	0
0.001	7	5.7	22
0.0025	16.5	14	46.5
0.005	31.2	26.2	71.6
0.01	57.3	36.6	92
∞	100	43.6	100

From the equations it can readily be seen that both the maximum and minimum rates are proportionate to p_b . Thus if p_b is increased n -fold, the values of t in Table IV must all be reduced n -fold so as to give the correct results, since as long as $p_b \times t$ is kept constant, the equations are numerically identical.

(II) For CO.

The results for CO are similarly shown in Table V and plotted in fig. 2.

Table V.—CO Uptake, expressed in per cent. Carboxyhæmoglobin.

<i>t.</i>	Maximum rate.	Minimum rate I.	Rate in solution.
	per cent.	per cent.	per cent.
0.00	0	0	0
0.01	15.3	14.5	22.0
0.02	30.0	27.0	39.5
0.04	54.8	46.0	63.2
∞	100	79.5	100

In this case the maximum rate solution is

$$\sqrt{y_0} - \sqrt{\bar{y}} < 5.18,$$

the first minimum rate solution is

$$y_0 - y > 0.453 \log_e \left[\frac{\cosh 1.33}{\cosh (1.33 e^{-12.5x})} \right] = 2.27 \times 10^{-3},$$

and the rate in solution is $dy/dt = -25y$.

In this case also the maximum and minimum rates are both proportional to p_b .

D. Consideration of Results and Future Experimental Work.

I. O_2 Uptake.—Within the first quarter of the process, the maximum and minimum solutions are close to one another, and should therefore furnish a good range for comparison between theory and experiment.

	second
Thus according to the maximum solution the time taken to reach	
25 per cent. O_2Hb	0.004
whilst according to the minimum solution the time taken to reach	
25 per cent. O_2Hb	0.005
Mean	0.0045

The mean time 0.0045 second must therefore be within ± 10 per cent. of the true solution. After this, however, the maximum and minimum curves begin to diverge rapidly and by the time 40 per cent. O_2Hb is reached, the mean of the two is only correct to within ± 30 per cent. Beyond this point the method breaks down altogether. Unfortunately, the best points obtained by Hartridge and Roughton (1927) in their experiments are almost all greater than 40 per cent. O_2Hb , and hence are of little or no value for comparison.

The controlling influence of diffusion within the corpuscle is however quite clearly established by these theoretical results, for inspection of fig. 1 shows that within the "good" range of 0-25 per cent. O_2Hb , the rate of the chemical reaction in solution is about four times greater than the calculated rate within the concentrated layer, and that above 40 per cent. O_2Hb the difference might well become larger. There is therefore no doubt that a large part of the discrepancy between the rate of $O_2 + Hb$ in solution and in corpuscle, as shown in Table I, is due to diffusion within the interior of the corpuscle.

In order to assess this factor still more exactly the following conditions must be satisfied in further experimental work :—

- (i) The *same* blood must be used in the solution experiments as in the corpuscle experiments.
- (ii) A method must be devised, accurate if possible to 1 per cent. or less of O_2Hb , of measuring the per cent. O_2Hb in the moving fluids within the range 0–25. The reversion spectroscope hitherto used is only

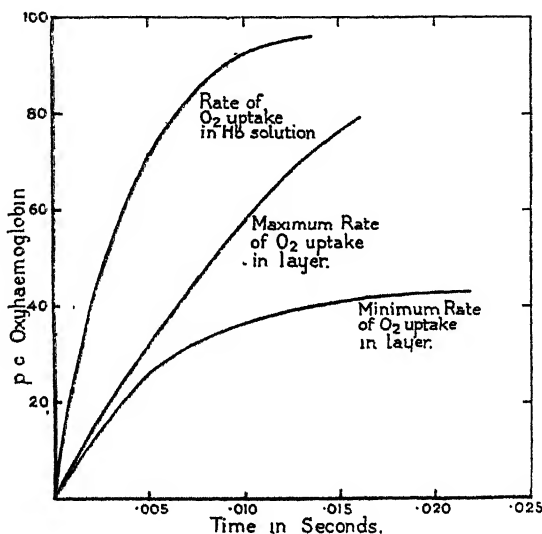


FIG. 1.

available in the range 35 to 65 per cent. O_2Hb , and even there is not good enough. Several kinds of technique, including photoelectric (Millikan and Roughton, 1931) and thermoelectric methods (Roughton, 1930), are being tested for this purpose.

- (iii) In order to eliminate the influence of diffusion of Hb within the corpuscle the p_{O_2} in the surrounding fluid should be as high as possible.
- (iv) In order to reduce or eliminate the influence of the back reaction $O_2Hb \rightarrow O_2 + Hb$ (neglected in the above treatment) the p_{O_2} should likewise be high, and the measurements should be made at $0^\circ C.$, since the back reaction would then be about 10 times slower than at room temperature (Hartridge and Roughton, 1923), whereas the forward reaction $O_2 + Hb \rightarrow O_2Hb$ would not be appreciably affected (Hartridge and Roughton, 1925).
- (v) If possible, the method should require that the samples of blood used

would be small enough to be applicable to man and to animals other than oxen, sheep and pigs.

All these points are being kept in view in the experimental work on which the writer is at present engaged.

II. *CO Uptake*.—In this case the maximum and minimum rate solutions are much closer to one another, and do not diverge by more than 20 per cent. until the process is half completed.

Therefore within the range 0–50 per cent. COHb, the mean of the maximum and minimum solutions must be a correct solution to within ± 10 per cent. or less. Fig. 2 shows that the rate of the chemical reaction is not more than 1.5 times faster than the calculated rate in the concentrated layer, so that the

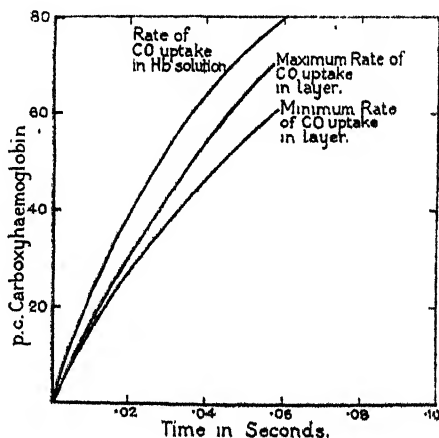


FIG. 2.

influence of diffusion within the corpuscle is in this case much smaller, though still significant. This agrees with the rough indication yielded by Section I, (C).

Further experimental work is required on the same lines as that just sketched for O_2 uptake. It may be possible to adapt the reversion spectroscope to the requirements of this case. There are two other respects in which this process is simpler and more satisfactory to study than the former one:—

- (i) Being slower, higher values of p_{CO} can be used.
- (ii) The back reaction, being 1000 or more times slower, can be neglected with complete safety.

E. Summary of Position Reached.

The final situation reached can now be summarised in Table VI, which is simply a new edition of Table III amended by the inclusion of the results obtained in Section II.

Table VI.

Process in corpuscle.	Extent by which diffusion through corpuscle membrane reduces rate as compared with rate in solution.	Extent by which diffusion within interior of corpuscle reduces rate as compared with rate in solution.
$O_2 + Hb \rightarrow O_2Hb$	Not known	75 per cent. or more
$CO + Hb \rightarrow COHb$	< 40 per cent.	33 per cent.
$O_2Hb \rightarrow O_2 + Hb$	< 70 per cent.	< 50 per cent.
$O_2 + COHb \rightarrow CO + O_2Hb$..	Negligible	Negligible

It is hoped that the study of $O_2 + Hb \rightarrow O_2Hb$, and $CO + Hb \rightarrow COHb$ on the same blood will throw further light upon the question of function of the corpuscle membrane.

The process $O_2Hb \rightarrow O_2 + Hb$ should also be within the range of the theoretical methods used in Section II, A, B, C, but the conditions are here quite different and may not prove so amenable to treatment.

Summary.

The factors determining the rate of uptake and release of O_2 and CO from the red blood corpuscle are three in number : Diffusion through the corpuscle membrane, diffusion within the interior of the corpuscle, and the chemical reaction inside the corpuscle.

In this paper an attempt has been made to decide which of these factors is responsible for the discrepancies between the rates of these processes in the corpuscle and in the homogeneous reaction in hæmoglobin solution.

In the case of the displacement of CO from Hb by O_2 , it is shown that both the diffusion factors are negligible, and since the rate of the process in corpuscle is experimentally the same as in solution, it is concluded that the chemical kinetics of the reaction are the same as in solution, and that the same is probably true of the other reactions also.

The function of the corpuscle membrane in the case of the other three processes is discussed. Arguments suggest that it may not be large, and maximum figures are given for its influence in the case of $CO + Hb \rightarrow COHb$ and $O_2Hb \rightarrow O_2 + Hb$.

Diffusion within the interior of the corpuscle is shown by preliminary calculation to be of great importance in the case of $O_2 + Hb \rightarrow O_2Hb$, but of less than 50 per cent. importance in the case of $CO + Hb \rightarrow COHb$, and $O_2Hb \rightarrow O_2 + Hb$.

Theoretical formulæ are then developed for the rate of uptake of O_2 and CO by a layer of hæmoglobin solution of the same concentration and thickness as the corpuscle. This is a heterogeneous process involving both diffusion constants and reaction velocity constants, and belongs to an important type not, so far as is known, hitherto worked out. The equations could not be solved exactly, but maximum and minimum solutions are obtained which are close enough to one another to enable the true solution to be reached to within a numerical accuracy of ± 10 per cent. over a considerable range for each process.

The application of those equations shows that diffusion within the layer (and hence presumably in the corpuscle also) cuts down the rate of O_2 uptake four-fold, and of CO uptake by one-and-a-half-fold, as compared with the rates of these processes in homogeneous solution, wherein diffusion is not a limiting factor.

Plans for further experimental work for describing these theoretical formulæ are discussed.

APPENDIX.

The diffusion constant of hæmoglobin in 30 per cent. solution, and its influence on the rate of uptake of O_2 and CO by a layer of 30 per cent. Hb .

In Section II it was pointed out that in calculating the rate of uptake of O_2 or CO by a layer of 30 per cent. hæmoglobin solution, it might be necessary to take into account not only the diffusion of O_2 and CO , but also the diffusion of Hb itself. In order to decide this matter it is necessary to know the diffusion constant of Hb in 30 per cent. solution. Unfortunately, there are no experimental data for the diffusion constant of Hb except in dilute solutions of 1 to 2 per cent. Hb in which, according to Northrop and Anson (1929),

$$\begin{aligned} D_{Hb} &= 0.042 \text{ per square centimetre per day} \\ &= 5 \times 10^{-7} \text{ per square centimetre per second.} \end{aligned}$$

A possible method of measuring the diffusion constant of Hb in concentrated solution has been devised by the writer, but it has not yet been possible

to put it into practice. A value which is probably a maximum one can, however, be calculated as follows.*

According to Anson and Northrop the diffusion constant of Hb in 1 to 2 per cent. solution obeys Einstein's Law, viz.,

$$D_{\text{Hb}} = \frac{RT}{N} \frac{1}{6\pi r\eta}$$

where R equals the gas constant in ergs, T the absolute temperature in degrees C., N the 6.06×10^{23} , r the radius of Hb particle in centimetre, and η the viscosity of water in C.g.s. units.

In concentrated solution Einstein's Law must be modified for two reasons:—

- (i) The osmotic pressure of the Hb is no longer proportional to its concentration, but rises much more rapidly than the latter. Experimental data by Adair (1929) show that at 30 per cent. Hb concentration, a correction factor of 3.8 is necessary, i.e., osmotic pressure at 30 per cent. concentration equals $RTC_p \times 3.8$ instead of RTC_p , where C_p equals molecules Hb per litre of solution.
- (ii) As pointed out by Cunningham (1910), when the particles are close together, the movement of a particle of the disperse phase encounters a much higher viscous resistance from the surrounding medium, for an equal volume of the latter has to be displaced and this must be squeezed through the spaces between adjacent particles of the disperse phase, and when these are close together the viscous forces increase enormously.

Cunningham has allowed for this factor by means of a correction to Stokes' Law.

When the disperse particles are far apart, the ordinary form of Stokes' Law is

$$F \text{ (viscous resistance)} = 6\pi\eta rv \text{ (velocity of movement of particle).}$$

Cunningham's corrected form runs

$$F = 6\pi\eta rvq,$$

where

$$q = \frac{4S(S^5 - r^5)}{(S - r)^2 [4S^4 - S^3r - 6S^2r^2 - Sr^3 + \dagger 4r^4]},$$

r = radius of particle

$$S = \sqrt{\frac{3}{2}}b$$

b = half the mean distance between the centre of adjacent particles.

* I am indebted to Dr. Eric Ponder for suggestions in this connection.

† In the original paper there is a misprint, this sign being given as — instead of +.

According to Cunningham, this value of q is probably an upper limit.

For 30 per cent. Hb solution, assuming that the density of the individual particles is the same as that in bulk, namely, 1.33 , $S/r = 1.57$ and $q = 33.5$.

In 1 per cent. Hb solution $q = 1.8$.

The influence of these two factors upon Einstein's Law can be readily worked out.

Consider first the case in which Einstein's Law holds.

The deduction of Einstein's Law can be made thus. Let n equal number of disperse particles at a point x , and $n + dn$ equals number of disperse particles at a point $x + dx$.

$$\text{Osmotic pressure at } x = P = \frac{RT}{N}n \text{ and at } x + dx = \frac{RT}{N}\left(n + \frac{\partial n}{\partial x}dx\right).$$

The number of particles which diffuse through unit area at x in time dt

$$= D \frac{\partial n}{\partial x} dt \text{ by the definition of } D.$$

The force on each particle between x and $x + dx$

$$\begin{aligned} &= \frac{\text{Difference in osmotic pressure at } x \text{ and } x + dx}{\text{Number of particles in layer of area 1 sq. cm., depth } dx}, \\ &= \frac{RT}{N} \frac{\partial n}{\partial x} dx \div n dx, \\ &= \frac{RT}{Nn} \frac{\partial n}{\partial x}. \end{aligned}$$

Therefore the velocity by Stokes' Law

$$= \frac{RT}{Nn} \frac{\partial n}{\partial x} \frac{1}{6\pi r \eta}.$$

Therefore the number of particles passing through unit area in time dt at x

$$\begin{aligned} &= n \times dt \times \frac{RT}{Nn} \frac{\partial n}{\partial x} \frac{1}{6\pi r \eta} \\ &= \frac{RT}{N} \frac{1}{6\pi r \eta} \frac{\partial n}{\partial x} dt, \end{aligned}$$

therefore

$$D = \frac{RT}{N} \frac{1}{6\pi r \eta}.$$

Now in 30 per cent. Hb solution $P = RTn/N \times 3.8$ (Adair's formula)

$$F = 6\pi r \eta v \times 33.5 \text{ (Cunningham's correction factor).}$$

Therefore by the same procedure D_{Hb} for 30 per cent. Hb solution

$$= \frac{RT \times 3.8}{N} \times \frac{1}{6\pi r \eta \times 33.5}.$$

For 1 per cent. Hb solution there is no correction factor for the osmotic pressure but $q = 1.8$.

$$D_{\text{Hb}} \text{ for 1 per cent. Hb solution} = \frac{RT}{N} \times \frac{1}{6\pi r \eta \times 1.8}.$$

Therefore

$$\frac{D_{\text{Hb}} \text{ for 30 per cent. solution}}{D_{\text{Hb}} \text{ for 1 per cent. solution}} = \frac{3.8 \times 1.8}{33.5} = 0.2,$$

because

$$D_{\text{Hb}} \text{ for 1 per cent. Hb} = 5 \times 10^{-7},$$

therefore

$$D_{\text{Hb}} \text{ for 30 per cent. Hb} = 1 \times 10^{-7},$$

which is probably an upper limit. When converted into the same units as were used for O_2 and CO it is found that

$$\frac{D_{\text{Hb}} \text{ in 30 per cent. solution}}{D_{O_2}} = \frac{1}{123}$$

$$\frac{D_{\text{Hb}} \text{ in 30 per cent. solution}}{D_{\text{CO}}} = \frac{1}{90}.$$

The Influence of Diffusion of Hb.

(i) On the rate of O_2 uptake.

Let $p_a = 75$ mm. as in Table IV.

At the end of 0.002 second the per cent. of $O_2\text{Hb}$ on the edge of the corpuscle where the chemical reaction proceeds unchecked is from fig. 1 = 40 per cent. $O_2\text{Hb}$.

The average per cent. of $O_2\text{Hb}$ over the interval 0 to 0.002 second = 20 per cent. $O_2\text{Hb}$ approximately.

The average extent to which the corpuscle would become saturated with O_2 during the 0.002 second, by virtue only of the diffusion of $O_2\text{Hb}$ from the periphery is, by equation III

$$= 20 \text{ per cent. } O_2\text{Hb} \times 0.25 = 5 \text{ per cent.}$$

This is only about one-third of the extent (*vide* Section II) to which the corpuscle is calculated to saturate by virtue of O_2 diffusion alone.

If $p_{O_2} = 750$ mm., and a time interval of 0.0002 second is considered—

O_2 Hb diffusion alone would bring about a saturation of 1.7 per cent.

O_2 diffusion alone according to table a saturation of 15 per cent.

so that in this case the influence of Hb diffusion only amounts to 1 in 10.

(ii) On the rate of CO uptake.

	per cent.
If $p_{CO} = 75$ mm. average per cent. of COHb by COHb diffusion alone	6
average per cent. of COHb by CO diffusion alone ..	15
Ratio.....	40
<hr/>	
= 750 mm. average per cent. of COHb by COHb diffusion alone	2
average per cent. of COHb by CO diffusion alone ..	15
Ratio.....	13
<hr/>	

Thus, as stated in Section II, the influence of Hb diffusion is very unlikely to cause an error of more than 10 to 30 per cent. even if there is no "structure" inside the corpuscle.

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*The Relation of the Duodenal Mucosa to the Internal Secretion
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Evidence that changes in the duodenal mucosa are present in diabetes mellitus is given by C. Workman in a private communication to Monroe (1906). A recent examination of Workman's autopsy protocols, in cases of diabetes, shows that hypertrophy of the duodenal mucosa and valvulæ conniventes was present in 10 out of 21 cases. The discovery of secretin by Bayliss and Starling and its striking effects on the external secretion of the pancreas led Moore, Eadie and Abram (1906) to postulate that the duodenum also elaborated a hormone that excited the internal secretion of the pancreas. They administered acid aqueous extracts of the duodenal and upper intestinal mucosa to three cases of diabetes and found that in two cases the urine became sugar free. Bainbridge and Beddard (1906) were unable to confirm these findings and ascribed the favourable results obtained by Moore and his collaborators to dietary control.

Following the discovery of insulin, several attempts were made to recover insulin from the duodenal mucosa. Ivy and Fisher (1924) isolated an insulin-like substance from the gastric and duodenal mucosa and Dixon and Wadia (1926) observed that extracts of duodenal mucosa injected into dogs acted in a manner similar to insulin in that both produced an increase in the output of pituitrin.

The presence of an insular hormone in the duodenum, the production of which is caused by presence of sugar in the intestine, is postulated by Macallum (1929), who holds that diabetes may result from insular fatigue due to excessive stimulation of the islets of Langerhans by this hormone. Heller (1929) showed that extracts of duodenal mucosa injected into normal rabbits prior to the injection of 0.5 gm. of glucose per kilogram body weight prevented as marked a hyperglycæmia as in rabbits not so treated, and also caused the blood sugar to return more rapidly to normal or subnormal levels. He (1931) also obtained similar results with dogs when his active principle (which he calls duodenin) was administered by the mouth.

Assuming that such a hormone exists and, as indicated above, previous work was inconclusive in that it did not demonstrate the existence of a hormone apart from insulin and secretin, the present work was undertaken. Preliminary reports of this work were published, Laughton, Macallum *et al.* (1930, 1931).

Methods.

The active material can be obtained from the duodenal mucosa of rabbits, dogs, pigs and cattle, but early in our work we found it necessary to use large quantities of mucosa which were supplied to us through the co-operation of a large meat-packing plant. The preparation used in the present work was prepared from the duodenal mucosa of cattle. It is essential that the duodenal mucosa be scraped off and frozen preferably with dry ice or carbon dioxide snow immediately after the animal is killed. It must be kept frozen during storage and transport. The material which has thawed out for any length of time prior to extraction has been found to be useless. It is also essential that material should be desiccated as rapidly as possible, on large steam trays using dry steam at about 40 lbs. pressure. The material must be spread in thin layers over the tray so that desiccation of the amounts described below is accomplished in approximately 1 hour's time. Evaporation over water baths prolongs the drying and tends to inactivate the material. While the following protocol was carried out in the laboratory, we have found that, through the co-operation of the packing house and an industrial corporation, which has a large plant for rapid desiccation of food materials in vacuum, the preparations with which they have furnished us have been far more active than any we have been able to prepare in the laboratory. The above conditions are essential to the preparation of material with any degree of activity.

6.7 kilos. of moist mucosa were desiccated, giving a yield of 1.1 kilogram of dried mucosa of the consistency of coarse bread crumbs. This was agitated for 3 hours with 5.5 litres of 95 per cent. ethyl alcohol to which 54 c.c. of concentrated hydrochloric acid had been added. After the agitation, the mixture was allowed to stand overnight at room temperature, the alcoholic fraction separated, and alcohol removed by distillation, leaving a residue of about $\frac{1}{2}$ litre of fatty material. This residue was agitated with 1 litre of 1 per cent. hydrochloric acid for 2 hours, and the acid solution separated and clarified with charcoal. This was again filtered, 50 grams of calcium phosphate were added to the filtrate and enough hydrochloric acid to bring about a solution of phosphate. Saturated sodium carbonate solution was added until a p_{H} of

7·8 to 8 was reached, when the calcium phosphate precipitates out and brings down with it, by coprecipitation, the major portion of the active material. The phosphate precipitate is filtrated off and sucked dry but not washed, placed in a hydraulic press and pressed to a cake, then dried by vacuum desiccation and ground to an impalpable powder. The yield of phosphate and coprecipitate varies from batch to batch, as each duodenal extract has a certain amount of naturally occurring phosphate in it which comes down when the acid is neutralised. In 50 grams of the precipitate approximately 10 grams are organic material containing the active duodenal principle. Of the organic residue obtained there is an unknown amount of extraneous material and further work in connection with the purification of this organic fraction has been in progress for some time. The experiments hereinafter described were carried out with the calcium phosphate precipitate, suspending 1 gram of precipitate in 20 c.c. of distilled water or 0·3 per cent. hydrochloric acid which dissolves nearly all the active material from the powder. The dissolved portions were used in the experiments described below. This preparation withstands sterilisation for 15 minutes at 250° F. and can be boiled in strong hydrochloric acid, but deteriorates upon standing in alkaline solution. The powder maintains its activity for months when kept at low temperatures.

Animal Experiments.

Rabbits and dogs were used for testing the preparations.

A. Experiments on Rabbits.—Tests for potency of extracts were made on rabbits. The test depended on the ability of the extract to reduce an artificial hyperglycæmia in healthy adult rabbits. The rabbits were starved for 24 hours prior to the experiment. A sample of blood was taken from the ear vein and immediately thereafter 1 c.c., per kilogram weight, of a 1 in 20 solution of the white powder was injected subcutaneously. After 30 minutes had elapsed a second sample of blood was withdrawn and glucose (0·5 gm. per kilogram body weight) injected into the vein of the other ear. Blood samples were now taken every 10 minutes until the end of the experiment. The blood sugar estimations were done by the method of Pickardt and Pierce (1920). Control experiments were carried out on other rabbits which received only the glucose intravenously. In this group 60 control experiments were done and 100 experiments testing the effects of the duodenal preparations. The results published are typical of all the experiments done.

In order to study the effect of prolonged administration of the extract a

series of four experiments were done. Rabbits were given subcutaneous injections of the duodenal preparation daily for 7-10 days. Following this course of injections, experiments were carried out at intervals in which glucose was administered intravenously and the time taken for the blood sugar to return to normal was estimated.

The effects of the duodenal preparations on the blood pressure of the rabbit and dog were studied. Blood pressure records were obtained from the carotid artery by the direct method. The effects of the duodenal preparation on adrenalin hyperglycæmia in a series of four rabbits were also studied.

B. *Experiments on Dogs.*—(a) Experiments were done on six normal dogs similar to those described for rabbits. In these experiments the blood was withdrawn from the saphenous vein and the glucose administered *per os*. The duodenal preparation was administered either subcutaneously or by mouth. The dogs were starved 36 hours prior to the experiment.

(b) In a series of four experiments, the effect of the preparation on the hyperglycæmia in two totally depancreatized dogs was studied.

(c) Two dogs were partially depancreatized and sufficient time allowed to elapse for the blood sugar to return to normal levels. In a series of 10 experiments on these two animals the effects of the duodenal preparation, on the hyperglycæmia produced by the oral administration of glucose, were studied under the following conditions: when (a) duodenal preparation was administered subcutaneously, (b) duodenal preparation was administered by the mouth. Control experiments were done in these animals prior to partial pancreatectomy as described for experiments on normal dogs.

A. Rabbits.

Observations.

I. *Effect of Extract on Blood Sugar of Normal Rabbits.*—The duodenal preparation has no hypoglycæmic or hyperglycæmic effects during the experiments which lasted for several hours.

II. *Effects of Duodenal Preparation on Artificial Hyperglycæmia.*—The normal blood sugar curve in a rabbit following the intravenous injection of 0.5 gm. glucose per kilogram weight is shown in fig. 1, graph 1. Graphs 2 and 3 show the blood sugar curve in rabbits, following the intravenous injection of 0.5 gm. glucose per kilogram body weight, which had received a small subcutaneous injection of the duodenal preparation 30 minutes prior to the injection of glucose. It will be noted from these graphs that in rabbits treated with the duodenal preparation the blood sugar did not attain the high level shown in the control, and also that the blood sugar returned to normal in

20 minutes, whereas in the control animal the normal level was reached only after a period of about 2 hours.

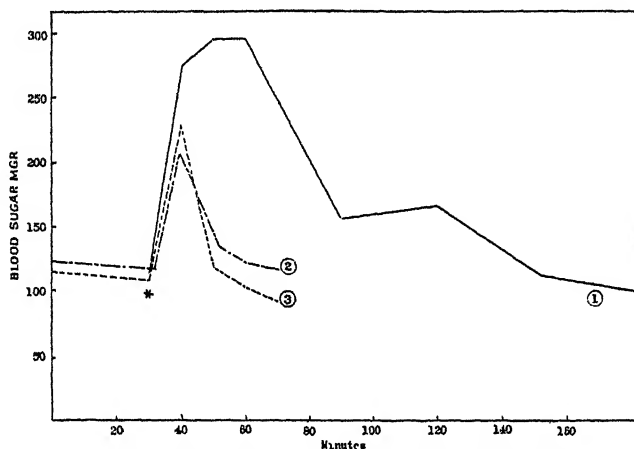


FIG. 1.—Effects of duodenal preparation on artificial hyperglycæmia in rabbits. Graph 1 is the blood sugar curve in a rabbit following the injection of 0.5 gm. glucose per kilogram weight. Graphs 2 and 3 show the blood sugar curves in rabbits following administration of 0.5 gm. glucose per kilogram weight, which received a small subcutaneous injection of the duodenal preparation 30 minutes prior to the administration of sugar. × point where glucose was injected.

III. The Effects of the Duodenal Preparation on Adrenalin Hyperglycæmia.—

Two rabbits were used in each experiment. One animal received a subcutaneous injection of the duodenal preparation and 1 c.c. of 1/10,000 adrenalin solution per kilo. The other rabbit received 1 c.c. of 1/10,000 adrenalin per kilo but none of the duodenal preparation. The blood sugar records are shown in fig. 2. Graph 2 represents the blood sugar curve in the rabbit

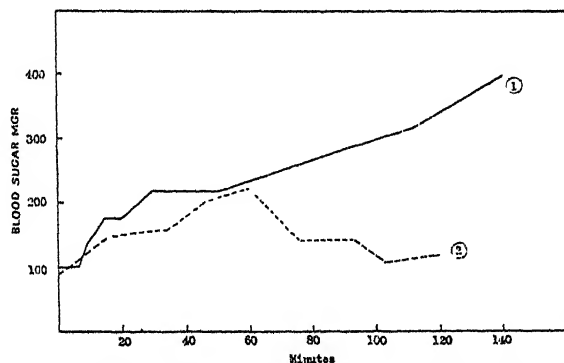


FIG. 2.—Adrenalin hyperglycæmia in rabbits. Graph 1, rabbit not treated with duodenal preparation. Graph 2, rabbit treated with subcutaneous duodenal injection.

treated with the duodenal preparation, while graph 1 shows the course of the blood sugar in the control animal. Here again the effect of the duodenal preparation is shown in the marked lowering of the blood sugar as compared with the control, which shows a steady increase throughout the experiment. The effect here is not so marked as that observed in animals in which glucose was given intravenously. This is probably due to the continuous output of glucose from the liver over a prolonged period as shown in the control animal.

IV. *The Effect of Prolonged Administration of the Duodenal Preparation on Blood Sugar.*—In animals, which had received daily administration of the duodenal preparation over a period from 7 to 10 days, it was observed that the effects of the substance on artificially induced hyperglycæmia persisted for a period of 2 weeks following the last injection.

Effect of Duodenal Preparation on Blood Pressure in Rabbits.

Intravenous injections of the preparation in rabbits had no effect on blood pressure.

Experiments on Dogs.

(a) *Normal Dogs.*—Fig. 3 indicates the effect of the duodenal preparation on artificially induced hyperglycæmia in the normal dog. Graph 1, fig. 3, represents the blood sugar curve of a dog which has received intravenous sugar only. Graph 2 represents the blood sugar curve for a dog which

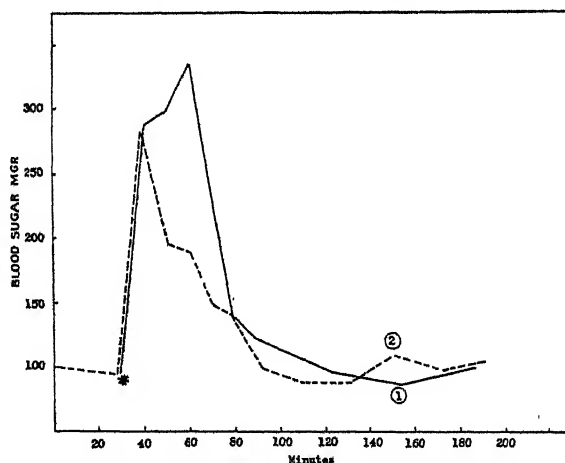


FIG. 3.—Effects of duodenal extract on artificial hyperglycæmia in normal dogs. Graph 1 represents blood sugar curve in untreated animal. Graph 2 portrays course of blood sugar in animal which received subcutaneous injection of duodenal preparation 30 minutes prior to sugar administration. ×, sugar administered intravenously.

had been treated with duodenal preparation 30 minutes prior to administration of glucose. Here again in the treated dog, as in the treated rabbit, the blood sugar did not reach the high level of the control and returned to normal more rapidly.

(b) *Partially Depancreatized Dog.*—In the partially depancreatized dog 2 months after operation, the effects of the duodenal preparations are shown in fig. 4. Graph 2 shows the hyperglycæmia induced by oral administration of glucose. It will be noted that hyperglycæmia was evident in a very few minutes following the administration of glucose and that the blood sugar persisted at a high level (about 500 mgm. per cent.) for the entire period. The effect of the duodenal preparation in controlling the hyperglycæmia is shown

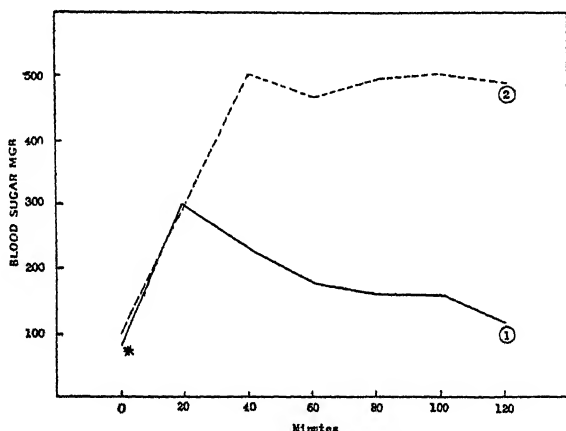


FIG. 4.—Partially depancreatized dog. Graph 2 shows the hyperglycæmia induced by oral administration of glucose. Graph 1 shows the blood sugar curves in dog treated with subcutaneous injection of duodenal extract 30 minutes prior to oral administration of glucose. ×, sugar administered orally.

in graph 1, fig. 4, when the preparation was administered subcutaneously 30 minutes prior to giving the glucose *per os*. The effect is more marked than in normal animals when the glucose tolerance is unaffected by operative interference. When the duodenal extract is given by mouth it has the same regulating effect on the hyperglycæmia produced by the oral administration of glucose as when it is given subcutaneously. Fig. 5, graph 1, shows the blood sugar curve in an animal which had received the preparation *per os* 140 minutes prior to the oral administration of the glucose. Graph 2 shows the blood sugar curve in same animal following the oral administration of glucose.

(c) *Effect of the Duodenal Preparation on the Totally Depancreatized Dog.*—The blood sugar values on a totally depancreatized dog treated with the

duodenal preparation are not reduced, and it is to be noted that following the injection the blood sugar values increased. This observation was made repeatedly in the totally depancreatized animal. In no case was a fall in the blood sugar level observed.

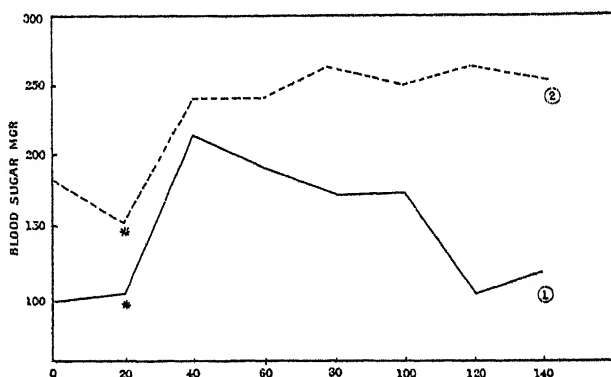


FIG. 5.—Partially depancreatized dog. Graph 1 represents blood sugar curve in animal following oral administration of duodenal preparation 140 minutes prior to oral administration of glucose. Graph 2, blood sugar curve in same animal following oral administration of glucose alone. X, glucose administered orally.

Relation of the Duodenal Preparation to Secretin.

Secretin preparations were made by the method of Bayliss and Starling (1902). The effects of secretin as compared with the effects of the duodenal preparation on the flow of pancreatic juice are shown in the following protocol:—

Normal rate of flow of pancreatic juice from pancreatic duct	1 drop in 2 minutes.
Rate of flow after injection of 10 c.c. of a 5 per cent. solution of duodenal preparation ..	1 drop in 2 minutes.
Rate of flow after injection of 10 c.c. secretin preparation	30 drops in 2 minutes.

Here the failure of the duodenal preparation to exert any effect on the flow of pancreatic juice is clearly demonstrated, indicating that it is entirely distinct from secretin.

Discussion.

A survey of the above recorded results would indicate that we are dealing with a substance in the duodenal mucosa which is a distinct entity and shows

its physiological activity by modifying artificially induced hyperglycæmia. Since it is obtained from the duodenal mucosa, secretin is a factor to be considered, but the lack of effect on the flow of pancreatic juice indicates that it is distinct from secretin. Furthermore, Mellanby (1928), Still and Shpiner (1929) have shown that pure secretin has no hypoglycæmic effect.

The hypothesis of a duodenal hormone governing the internal secretion of the pancreas previously postulated by Moore, Eadie and Abram (1906) and Macallum (1929) would seem to be substantiated by our experiments. We have shown that in the totally depancreatized dog the duodenal preparation produced no fall in the blood sugar, whereas in the partially depancreatized dog the preparation prevented the marked hyperglycæmia which occurs on the oral administration of glucose and, furthermore, caused the blood sugar to return rapidly to the normal resting level. It should be emphasised that, in none of the experiments did the duodenal preparation reduce the blood sugar below the normal resting level after an induced hyperglycæmia nor does it affect a normal blood sugar. This, combined with the fact that blood sugar levels in depancreatized dogs were unaffected, indicates that this material is distinct from insulin.

As to the mode of action of our preparation it would appear that the most probable explanation lies in the assumption that the preparation stimulates the islets of Langerhans to secrete insulin. If this is correct there must be a very delicate balance or the insulin, if in excess, would tend to produce a hypoglycæmia. Our observations gave no evidence that this occurs.

It is doubtful if the storage of sugar in the liver or kidney output is increased as the blood sugar in the totally depancreatized dog was not lowered by the extract. If our theory is correct, the utilisation of sugar would be similar to that following the administration of insulin.

Summary.

(1) An active preparation has been isolated from the duodenal mucosa of rabbits, dogs, hogs, and cattle which is distinct from secretin and insulin.

(2) This material has no hypoglycæmia effect in normal animals (rabbits and dogs).

(3) The active principle controls experimental hyperglycæmia in normal animals.

(4) The active principle has no effect on the hyperglycæmia in totally depancreatized dogs.

- (5) The active principle lessens the hyperglycæmia induced by oral administration of glucose in partially depancreatized dogs.
- (6) The probability that the substance is an insular hormone is discussed.

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The Morphology and Cytology of Bacterium malvacearum, E.F.S.
Part II.—Reproduction and Cell-Fusion.

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[PLATES 1 AND 2.]

In an earlier paper (Stoughton, 1929) an account was given of certain observations on the morphological and cytological changes undergone by *Bacterium (Pseudomonas) malvacearum*, E.F.S., the causal organism of the angular leaf-spot disease of cotton plants.

A central, deeply-staining body was demonstrated in the bacterium, and by means of a special staining technique the changes through which it passes were traced. The structure divides simultaneously with the division of the cell-body and the method of this division was described. Further evidence was

adduced tending to show that this body is of the nature of a true bacterial nucleus or, alternatively, a nucleus embedded in a matrix of "chromatic" material. The formation and liberation of very small deeply-staining bodies, which appear to be identical with the "gonidia" of other workers, were described.

An account was also given of the method of production of spherical coccus-like bodies, formed by a process analogous to the budding of yeasts, which appear to constitute a method of vegetative reproduction not previously described for the organism. At the time the previous paper was written the subsequent development of these "cocci" had not been traced, but further work has thrown light on this point.

Throughout the work a single strain of *B. malvacearum* of proved virulence has been used. The strain has been kept in a state of assured purity by constant re-plating and occasional re-starting from a single cell isolated by means of the Dickinson micro-isolator (1926). The cultures have been grown throughout on potato-extract agar containing 1 per cent. of sucrose and their virulence maintained by frequent passage through cotton plants. All cultures were kept for at least 1 week in an incubator at 25° C. and then transferred to an unheated storage chamber.

The technique employed in preparing the slides was given in the previous paper, but further work with the method has shown certain points worthy of note. A common source of failure has been the use of too much dye solution in preparing the film of stain. Experience alone will show how much stain is necessary for a good result with any particular organism or culture, but the amount will always be small. The film should be barely perceptible when held to the light, and the final preparation should show the nuclear-like bodies strongly stained and the outer membrane distinct, but the remainder of the cell almost unstained. A second source of failure is the use of too thick a water-film. Using cover-glasses of 2 cm. diameter a loopful of water from a 2 mm. platinum loop just fills the space between the cover and the slide without excess, forming a film approximately 5-10 microns thick. One other point is that slides prepared by this method are in no sense really permanent, but remain good for about 2 days only. After this period granulation of the contents of the cells occurs and entirely misleading appearances may be seen.

For routine examination of the slides a high-power water-immersion objective is a convenience as the covers are not soiled and, if necessary, the slides may be examined repeatedly during the day or two that they remain good. Such an objective is also more suited to the examination of objects mounted in a watery

fluid than an oil-immersion lens. For photography, or for critical examination of the finer details an objective with higher aperture and consequent greater resolving power is needed. In this case better results are obtained with the organisms which are in contact with the cover-glass than with those lying on the slide and viewed through the water-film. The latter interferes with the corrections of the objective, though some improvement may be effected by suitable extension of the tube-length. If, however, the film exceeds about $10\ \mu$ in thickness, a satisfactory image of the organisms on the surface of the slide cannot be obtained with an oil-immersion lens. The presence of the water-film reduces the working aperture in any case to a maximum of about 1.3, but this is sufficient for a clear resolution of the main details.

Life-cycle of the Cocci.

Certain stages in the production of the coccoid bodies were described in the previous paper, but fuller details of the earlier stages and of the subsequent development have now been obtained. Figs. 1-3, Plate 1, show the first stages in the formation of the bud, before the division of the nucleus in the parent cell. Fig. 4 on the same plate shows the beginning of this division, the later stages of which were given in figs. 3 and 4, Plate 26, of the previous paper. Owing to the optical limitations discussed in the earlier paper and the considerations referred to above, the minute details of the division cannot be finally determined. The process appears to be a "pinching in two" of the "chromatin" material more or less coincidently with the abscission of the neck joining the coccus to the parent rod. In fig. 5, Plate 1, the coccoid body has attained its full size and is on the point of liberation. The coccus now becomes free in the medium, and in a suitable culture large numbers of the free cocci can be seen, each with its single deeply-stained nucleus-like body. These coccus forms are extremely thin-walled, especially while still growing attached to the parent cell, and in consequence are very easily distorted or destroyed. This fact explains the failure to see the structures in dried-film preparations. Even in a well-stained wet-film preparation critical optical conditions are essential for a clear picture of the formation.

After an interval, as yet undetermined, the cocci germinate. A small papilla appears at one point and this grows out into a rod, apparently identical with the normal vegetative cell, figs. 6, 7 and 8, Plate 1. So far, the cytological changes associated with the germination have not been determined. In this stage the cell appears to contain a large amount of food-material which stains rapidly and deeply, the dense stain rendering it difficult to make out the

structure. The behaviour is similar in this respect to that of normal vegetative cells from a very young culture, where, as noted in the previous paper, the dense staining rapidly obscures the internal structures.

The development of these bodies seems, therefore, to follow a closed cycle comparable with the vegetative spore-cycle of the lower fungi.

Cell-fusion and "Zygospore" Formation.

In the previous paper reference was made to the occurrence in old cultures (3-6 weeks or more) of characteristic "angled" forms, consisting of two cells apparently united at one end and forming an obtuse angle to one another, fig. 9, Plate 1. It was suggested at that time that this formation might possibly represent an incomplete but more or less normal vegetative division, but further observations have failed to confirm this, and indicate rather that the appearance represents the first stage in the fusion of two independent cells uniting by their extreme ends. A number of forms have been repeatedly observed which fit into a series interpretable as stages in the production of a fusion-cell or "zygospore," and which are difficult to explain on any other basis.

At times the pairs are united by an unmistakable bridge or neck of variable length, fig. 9, Plate 1, but in most cases an obvious tube is not present, and the connection appears to be formed by a breaking-down of the wall of each cell at the point of contact. Here a small swelling appears, very similar in its early stages to the coccoid bodies previously described, fig. 10, Plate 2. This protuberance is at first very thin-walled, and stains only lightly, but at once begins to thicken its walls; the contents become denser and more deeply staining, while, usually, the parent cells become distinctly less dense. The newly-formed body attains a diameter equal to, or rather greater than, the width of the parent cells, figs. 12, 13, 14 and 15, Plate 2. The whole structure stains deeply at this stage, having, as a rule, considerably more affinity for the stain than the vegetative rods. Here again the cytological processes are difficult to determine. In the early stages, where the cells are joined at the tip, the two "nuclear" bodies of the joined cells lie near the point of junction, fig. 10, Plate 2, but in the later stages it is often possible to see the "nuclei" in the middle of each of the subtending cells, fig. 12, Plate 2.

The subsequent history of these spherical spore-like bodies has not yet been determined with certainty. The frequent appearance of two unequal "arms" in the fully-formed structure, fig. 16, Plate 2, where one "arm" seems to be

undergoing a process of degeneration, suggests that after the structure has attained its full size the parent cells shrivel and fall off. This inequality of the "arms" was at first thought to be due to the production of the spore-like body from the fusion of two cells of originally different sizes, but in all cases so far observed, when the "fusion-cell" is not full-grown, the subtending cells are equal in size, indicating that the difference is due to subsequent unequal shrivelling of the parent cells. In fig. 15, *b*, Plate 2, the parent cells are shrivelling simultaneously. The "zygospores" when free are similar in size and shape to the vegetatively-produced "cocci," but may in some preparations be distinguished by their affinity for the stain. The fusion-bodies during the course of their development acquire a power of staining strongly and appear as dense spherical cells in which no structure is visible, while the cocci usually stain much more lightly, and this distinction is maintained after liberation. When the coccoid bodies germinate, however, they also become easily and deeply stained. There appears therefore to be no way of determining by the observation of stained preparations alone whether the fusion-cells or "zygospores" subsequently germinate, since no culture containing "zygospores" but free from cocci has yet been obtained, although the latter may occur in the absence of the former.

Bodies similar to these zygospore-like forms have been observed in other plant-pathogenic bacteria, but their development in these cases has not at present been worked out in any detail. A striking example of such formation is given in fig. 18, Plate 2, which is taken from a culture sent as *B. stewarti* to the writer by Dr. A. J. Riker, of the University of Wisconsin. Several of the zygospore-like bodies are shown in one field. In these the subtending cells are nearly empty of stainable material although, in each, one very small granule, which may represent the nucleus, is discernible.

Many of the appearances observed in *B. malvacearum* closely resemble Mellon's figures of "zygospores" in *B. coli* (Mellon, 1925, 1926, 1927). More recently Stapp and Zycha (1931) have observed similar appearances in dried-film preparations of *B. mycoides*, but have interpreted them as artefacts produced by gross overstaining. That the bodies described for *B. malvacearum* cannot be so considered, has been demonstrated by their recognition in preparations of the living, unstained organisms. Three photomicrographs of such unstained cells are shown in figs. 11 and 17, Plate 2, under dark-ground illumination. Fig. 11 shows the early stage of "zygospore" formation immediately after fusion, while in fig. 17 the body is fully formed. The latter photograph shows the highly refractive nature of the mature "spore," which

is associated with its strong affinity for the stain in the wet-film preparations. Immature bodies are less refractive than the parent-cells, fig. 11, *b*, Plate 2, a fact which again agrees with the staining capacity.

Further evidence that these zygospor-like bodies are not artefacts is afforded by preparations made by the standard protozoological methods. Fig. 13, Plate 2, is taken from a cover-glass preparation fixed while still wet in hot Schaudinn's fluid, stained by Heidenhain's iron-haematoxylin process and mounted in balsam. The appearance is essentially the same as with the wet-film method.

Numerous attempts have been made to watch the various processes occurring under continuous microscopic observation. The difficulties inherent in such work were discussed in the previous paper, and they are reinforced in this case by the time factor involved in the germination of the cocci or "zygospores." Different methods have been tried, including the preparation of minute hanging drops by the use of the Chambers micro-manipulator, the observation of thin liquid films under dark-ground illumination, the hanging-block method, and the agar-film method (Stoughton, 1929). Partial success only has attended these experiments, growth of the rod from germinating cocci having been observed during a few hours. In nearly all cases where the conditions are such that growth can take place, the multiplication of the ordinary rods is so rapid that they soon overgrow the single cell under observation. Further, any interpretations placed on even apparently successful observations should be accepted with great caution, since the optical conditions under which such observations must be made, involving very considerable reduction in the numerical aperture of the illuminating cone, favour the production of false images. The exception would be observation under dark-ground illumination of high obliquity but, as pointed out in the previous paper, this method seems impossible of application in the case of *B. malvacearum*, which requires free access of air for growth.

Summary.

(1) Using a technique described in a previous paper, new morphological forms have been observed in *Bacterium malvacearum*.

(2) The production of coccoid bodies, their liberation, and subsequent germination to form apparently normal rods, are described.

(3) The formation of densely-staining spherical bodies, apparently arising from the point of fusion of two cells, is described. These bodies are apparently liberated by the degeneration of the parent cell.

EXPLANATION OF PLATES.

PLATE 1.

- FIGS. 1-5.—4-12 week cultures of *Bacterium malvacearum*, showing stages in the formation of the coccoid reproductive body. Wet-film preparations. Photographed on panchromatic plates with green filter. Leitz 2 mm. fluorite objective N.A. 1.32. $\times 10$ "periplanatic" ocular, Leitz aplanatic condenser, N.A. 1.40. $\times 2000$.
- FIGS. 6-8.—Stages in the germination of the "cocci" to normal rods. Figs. 6 and 7 photographed with Reichert 1/12-inch achromatic objective, other details as figs. 1-5. $\times 1600$. Fig. 8 as figs. 1-5. $\times 2000$.
- FIG. 9.—4-week culture showing early stage of fusion. Photographic details as figs. 1-5. $\times 2000$.

PLATE 2.

- FIG. 10.—4-week culture showing early stage of formation of zygospor. Photographic details as Plate 1, figs. 1-5. $\times 2000$.
- FIG. 11.—5-week culture mounted in sterile water. Photographed on panchromatic plate with green filter. Watson 1/12-inch achromatic objective with funnel stop (N.A. 0.95 approx.), $\times 10$ "periplanatic" ocular, Leitz dark-ground illuminator. (a) $\times 1400$, (b) $\times 2200$.
- FIG. 12.—4-8 week cultures showing formation of zygospor. Photographic details as Plate 1, figs. 1-5. $\times 2000$.
- FIG. 13.—4-week culture fixed in Schaudinn's fluid, stained iron-haematoxylin. Photographic details as Plate 1, figs. 1-5, a and b. $\times 2000$.
- FIG. 14.—8-week culture showing nearly mature zygospor. Photographic details as Plate 1, figs. 1-5. $\times 2000$.
- FIG. 15.—7-week culture showing (a) mature zygospor, (b) mature zygospor with parent cells beginning to degenerate. Photographic details as Plate 1, figs. 1-5. $\times 2000$.
- FIG. 16.—4-week culture showing mature zygospor and degeneration of parent cells. Photographic details as Plate 1, figs. 1-5. $\times 2000$.
- FIG. 17.—5-week culture in sterile water. Mature zygospor. Unstained, living forms, dark-ground illumination. Photographic details as fig. 2. $\times 2200$.
- FIG. 18.—2-week culture of *B. stewarti* with mature zygospor. $\times 1500$.

N.B.—All preparations are made by the wet-film process except fig. 13, Plate 2, which is a fixed preparation mounted in balsam, and the dark-ground figures, which are water-film preparations unstained.

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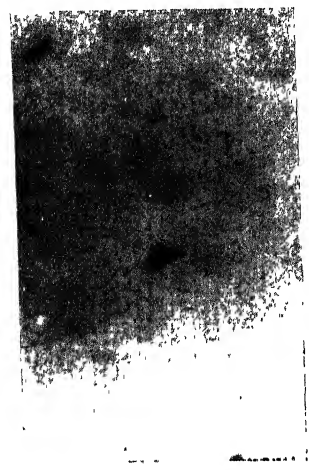
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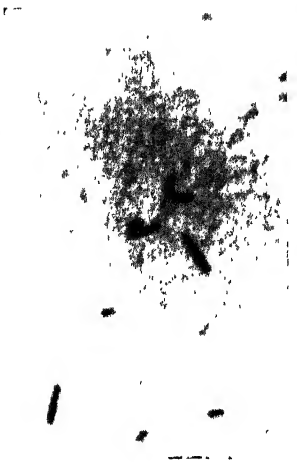
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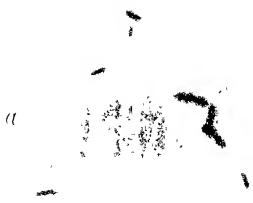
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An Analysis of some Necrotic Virus Diseases of the Potato.

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[PLATES 3 AND 4.]

Introduction.

The term "streak" has been applied somewhat indiscriminately to a variety of diseases of the potato in which the outstanding lesion is the appearance of necrotic patches in stem or leaf or both, such areas being more or less elongated. The first description of a disease of this type in the potato was made by Orton (1914) in 1912; a more detailed account of the same followed in 1920 (1920). Orton's description brings out certain salient points: in the diseases he describes the lesions are first seen in the adult leaves about one-third of the way down the stem as elongated spots which follow the veins and invade the parenchyma, and when viewed from the under surface of the leaf are seen to follow the veins as discoloured streaks. The disease spreads with great rapidity on the plant, the petioles become involved and collapse and, as a consequence, the leaf withers and hangs by a thread to the stem. The stem itself becomes brittle, turns brown, and dies at a point below the tip. The discolouration is superficial and apparently does not involve the vascular bundles. He observed no lesions in the tuber. Orton further noted the fact that whilst certain varieties such as Factor (Up-to-Date) were highly susceptible, many seedlings appeared to be immune. He could find no pathogene for this disease. Murphy (1921) described two conditions which he denoted as streak and leaf-drop; it would appear that the former is merely the seasonal, the latter the secondary form of one and the same affection.

Schultz and Folsom (1925) give a description of an affection in Green Mountain which corresponds closely to that given by Murphy for his streak. Atanasoff (1922, a) gave a full account of this same disease as exhibited in Duke of York and President. Most of his conclusions are based on the behaviour of the former variety, which is unfortunate, for, as we shall see, the Duke of York or Schottische Muis, as Atanasoff calls it, is a frequent carrier of another type of streak. Later, Atanasoff (1923—1925) mentions that this same streak could be transmitted by sap inoculation and by aphides, a characteristic of the streak.

under discussion. He (1922, b) has laid all workers under an obligation by his research into the history of the so-called degeneration diseases of the potato in general, and particularly in his attempt to track down this particular disease as the evil genius of the potato from 1775 onwards. Recent work which has shown this disease to be due to a single aphid-carried virus, adds much strength to this claim.

Quanjer and Botjes (1929) describe no less than eight various types of streak, four of which are of the leaf-drop type, and three belong to a different group to which they have given the name top-necrosis. The first four groups are named, one after Atanasoff and the remaining three after the varieties in which they are said to be most characteristic. These four groups are probably merely variants of the same disease induced by a single virus in varieties some of which are themselves carriers of other viruses; the fifth, sixth and seventh group are all examples of the streak referred to as top-necrosis, this time grouped according to the varieties in which they are latent. We owe these authors a debt of gratitude for bringing into relief the very real distinction between these two main types of streak and may ignore the unreal distinctions involved in their further sub-division, which have not only failed to obtain acceptance but have been completely superseded by later contributions of Quanjer (1931) himself. Here, recognising that the same virus will react quite differently on different varieties—a fact which had been already demonstrated by Salaman (1930, b)—Quanjer has introduced a classification of virus diseases based on the histological changes, more especially those which display the location and nature of the necrotic lesions. In this, Quanjer based himself on the valuable work of Artschwager (1923) who described streak lesions of the stem as proceeding acropetally in the vertical plane, and centripetally in the horizontal. In this last work Quanjer (1931) has built up a classification, on an anatomical basis, of potato virus diseases which exhibit necrotic lesions, which allows of the formation of six groups, three of which, viz., those dealing with phloem necrosis, phloem parenchyma necrosis of the tuber, and concentric necrosis of the tuber, we are not concerned with. A consideration of the remaining three groups in the light of new facts forms a considerable part of the subject matter of our communication.

Section 1 (Quanjer, 1931). Anecrotic Mosaic.—"No necrosis, no streak, no drop of the lower leaves of other varieties after they have been grafted with the virus-carrying scion; only mottling and more or less wrinkling of leaflets." Here are included "mild mosaic, intermediate mosaic,* crinkle mosaic or

* The writers are not aware of the significance to be attached to this term.

interveinal mosaic." They are both sap and aphid transmissible. X-bodies are present, striate material absent. Quanjer states that should one of these be found on communication to another variety to induce necrosis, it must be removed from this group.

This provision of Quanjer's illustrates the unreality of a classification based on anything else than the nature of the ultimate constituent virus actually at work. Kenneth Smith's (1931, *b*) work has shown that these diseases are not all aphid transmissible, but that only one constituent of them is so, and Salaman (1930, *b*) has shown that whilst they may be communicable by graft they are not so necessarily by sap infection; he (1932) has also shown that all these diseases will produce a streak on one or other variety.

Section III (Quanjer, 1931). *Top-necrosis—Acronecrosis*.—"Necrosis radiating from only a rather small percentage of the internal phloem strands, almost never from the external phloem strands, into the surrounding parenchyma, this in turn surrounded by a cork cambium, except in the tender tips which are soon killed; occurring in foliage, stem and tuber."

Section IV. Acropetal Necrosis.—"Necrosis chiefly in the collenchyma of the leaf veins, petioles, and stems, in cases extending gradually to other tissues, no restriction by a cork cambium. The advance along the stem is acropetal; dropping of lower leaves. Rugose mosaic and a part of the disease collectively known as crinkle and streak are characterised by this type of necrosis."

The data and conclusions here presented, support Quanjer's classification in respect of Groups III and IV, the former of which, however, must now be divided into at least four sub-sections.

The next advance in our knowledge, and by far the most vital, was the discovery and isolation by Kenneth Smith (1931, *a, b*) of the two unit viruses, X and Y. We shall be able to show that the major part, if not the whole of the acropetal necrotic group, is to be accounted for by simple infections with the Y virus, or, in certain cases, by the infection of this same virus superimposed on an existing virus complex carried by the plant in a latent condition. In this latter case the symptoms may be modified, as in Schulz's Green Mountain streak. Indeed, it is to the prevalence and unsuspected specificity of carriers that much of the confusion which has shrouded the subject must be ascribed.

We shall further be able to show that top-necrosis in some varieties is due to the action of the X virus alone, whilst in other varieties it is the sequela of a complex of which some of the constituents are known. The existence of a third virus element, Z, has been demonstrated by Salaman (1932), but the part

it plays in the composition of streak-producing complexes is in most cases still to be determined.

The survey of the literature to date allows us to distinguish two clearly differentiated clinical diseases under the headings of:—

A. Acropetal necrosis = stipple streak = leaf-drop streak.

B. Acronecrotic necrosis = Top-necrosis, Quanker, and the streak of some older writers.

As long as we bear in mind that both these names are employed as terms to distinguish actual clinical pictures and do not necessarily correspond to the reactions of specific unit viruses, they are of value; directly they are regarded as the inevitable reaction of a specific virus, or group of viruses, on the potato they are misleading and dangerous.

Our communication deals with these two clinical pictures—the behaviour of their causal virus or virus groups on different varieties and the demonstration that such groups may themselves be differentiated by these reactions.

A description of the histological character of the lesions will be found in a paper by F. C. Bawden (1932).

For comparison the two types of necrosis will be here described as exhibited on the variety President.

Acropetal necrosis as seen in the variety President. The following description is based on a large number of infections of President, both by inoculations and grafting with Kenneth Smith's Y virus; the symptoms do not in any way differ from those induced by Smith by infections with the same virus by means of *Myzus persicae*.

One stock of the Y virus in a Majestic seedling came direct from the cultures of Dr. Kenneth Smith, to whom we wish to express our grateful thanks. Another was from a plant of Epicure which was discovered by one of us in the field in 1930 and extensively studied. We had elucidated most of the reactions of this virus before we realised that the virus with which we were working was identical with the Y virus that Kenneth Smith had isolated earlier and described a little later.

The first symptom usually appears about 28 days after infection and consists of a blotchy mottle spreading from the veins and affecting the uppermost leaves only. This mottle later becomes intensified and is accompanied by some wrinkling and waving of the leaves, thus producing the picture of a crinkle. A little later necrosis appears on the underside of the veins of those leaves occupying an intermediate position on the stem, the upper surface of which may for a time look normal. These necroses increase in severity and spread

most rapidly along the course of the veins on the under surface of the leaf, appearing as elongated brown stripes on the petioles and as blotches between veins; later they penetrate the leaf tissue and become evident on the upper surface. The necroses pass down the petiole to the main stem and the leaf then collapses, rapidly withers, and remains hanging, as it were, by a thread, fig. 1, Plate 3.

Beneath the hanging leaves the stem frequently exhibits elongated brown stripes, but does not itself collapse as in the case of top-necrosis. This dropping of leaves following on the necrosis commences below and advances acropetally until in severe cases all the leaves except those at the very top may be seen hanging or have fallen to the ground. The topmost younger leaves rarely show any marked necroses, but they may become highly crinkled.

Infected plants die early and the tubers are of necessity few and small, but otherwise appear to be perfectly normal. Both necrosis and leaf-drop appear to be a first year or seasonal reaction, and though rather more striking and severe when the plants are grown under glass, are of the same general character when grown in the field. When the infection occurs naturally in the field as a result of aphid infection, symptoms do not make their appearance till well into July and the ensuing destruction is not so great as that which may be induced in the house. In their second year, under glasshouse conditions, after infection plants show but little sign of either necrosis or leaf-drop; they are small and stunted, their leaves and stems highly brittle; the internodes are short, and the leaves, generally mottled, are severely twisted and waved and bunched together, fig. 2, Plate 3. In the field, infected President plants in their second year present a picture which in extreme cases may resemble that of curly-dwarf.

Several infected plants have been examined for the presence of intracellular inclusions, or X-bodies, and none have been found.

Top-necrosis on President.—The first symptom which appears in about 15 to 20 days after grafting* consists of numerous minute irregular necrotic spots on the small folded leaves which make up the growing points, fig. 3, Plate 3. These necrotic lesions appear equally on veins and in the interveinal areas, rapidly increasing in size until by coalescing they destroy the small leaves and finally the whole top of the plant. The disease then spreads from above downwards, involving the stem as it goes. If the plant be young, it may be com-

* Not all top-necroses can be sap inoculated; indeed, if President be the test plant used, most cannot be so communicated. The acropetal necrosis can always be communicated by needle.

pletely killed out within a month. If, however, the plant is older, only the top and younger parts of the plant are killed outright. If the plant survives the first onslaught, further necroses may appear on the lower leaves as large blotches which penetrate the whole leaf tissue; they do not spread rapidly and the leaf may fall and wither, but does not hang in the characteristic manner of the Y infected plants, fig. 4, Plate 3. The absence of all mottling is a feature in the acronecrotic type in President which further distinguishes the two types of streak. Plants infected when young rarely produce any tubers. If such are formed the majority exhibit definite and distinctive lesions during storage, although normal to all appearance when harvested. Such tubers are small and mis-shapen, fig. 5, Plate 4; they exhibit deep fissures or ulcerous depressions. The eyes are frequently destroyed by similar necroses and consequently they rarely sprout in the following spring, fig. 6, Plate 4. On section, such tubers exhibit large darkly coloured necrotic areas which extend centripetally and frequently result in the entire tuber being converted into a more or less spongy, corky mass. Tubers which are normal, *i.e.*, develop no necroses, will sprout and develop normal and healthy plants; if a plant does grow from an infected tuber, it rarely develops, but when such does happen the resulting plant is dwarfed and extremely brittle, its leaves drop before they have unfolded, and the whole dies within a short time, fig. 7, Plate 4.

The descriptions here given for these two-streak types, as seen in President, agree closely with those given by Atanasoff and Quanjer; indeed, in regard to top-necrosis, we are in full accord with the latter author's description of the disease in President. There is, however, a difference between our findings in regard to the tuber condition in acropetal necrosis. Atanasoff pictures deep ulcerous depressions in corky mis-shapen tubers of affected Duke of York in their second season, and blister-like swellings and superficial erosions in the same variety in its first season. Quanjer depicts very superficial necrosis in otherwise normal tubers following a seasonal infection. We find no such lesions, and the reason may lie in the fact that we have infected our virus-free material with a single virus, *viz.*, Y, whilst Atanasoff, employing green-flies as vectors, unconsciously infected plants which were not virus-free but carried other viruses in addition to the pure Y virus, for it is this virus alone which the aphid transmits from a mixture. Quanjer, on the other hand, has used virus-free material as the subjects for infection, but his source of the virus is contaminated, *viz.*, Zeeland Blue, and his method of infection, *viz.*, core grafting, is particularly adapted to convey all the virus elements which may be present.

We may conclude that these two clinical types of virus disease differ in their mode of attack on both haulm and tuber.

We know of no causative agent for acropetal necrosis or leaf-drop streak other than the Y virus, but the reader is referred to Salaman's (1932) account of the reactions of that virus on varieties of the potato, from which it appears that leaf-drop streak is only developed in a few of our better known varieties, such as Arran Banner, Majestic, and Up-to-Date.

For further reactions on other Solanaceæ the reader is referred to Dr. Kenneth Smith's work (1931, *b.*).

The Acronecrotic or Top-necrosis Group of Streaks.

Just as we have seen that the Y virus will evoke a whole gamut of reactions according to the variety infected, so too does the X virus, but in this case the necrosis is typically acronecrotic. The reader is referred to the same source for a description of the reaction of the X virus on varieties of the potato. Out of 19 examined, 3 only respond with a top-necrosis, viz., Arran Crest, Epicure and King Edward; all the remainder display an interveinal mosaic.

For the reaction of this virus on other Solanaceæ, see Kenneth Smith (*loc. cit.*).

Top-necrosis—Section X.—Top-necrosis is seen in Arran Crest, Epicure, and King Edward. To take the last first: the X virus was inoculated into six plants, and all developed acute necrosis of the growing points in 15 days, which in most cases killed the plant. The tubers are either free of lesions or show the destructive necrosis and cork formation already described. In the second year the seed tubers either fail to sprout or produce healthy plants.

King Edward is a constant carrier of the Paracrinkle complex which contains the two virus entities Z and Y. Till we can obtain a King Edward free from this complex, we cannot say whether the top-necrosis developed is due to X itself or induced by a new complex which may possibly be formed by the interaction of the latent Paracrinkle and itself.

With Epicure and Arran Crest the case is, as far as we know, simple. We have stocks of either variety which we believe to be virus-free—or perhaps it would be more correct to describe them as stocks which have not yet been “found out.” In these, an acute necrosis of all the growing points sets in in 14 days and kills the plants. This has been observed five times in Epicure and three times in Arran Crest. Second season plants of both varieties have proved to be quite healthy when raised from tubers which were free of visible lesions.

In this section infection can be equally well induced by inoculation as by

grafting. No insect vector has so far been discovered. Intracellular inclusions or X-bodies occur in all the X-infected plants that have been examined which exhibit mosaic symptoms. In one top-necrosis due to the X-virus in Epicure, none were found; presumably the advance of the disease was too rapid for their formation.

In the course of the experimental work of the last four years, the senior author has found, as have other workers, a number of varieties, many individuals of which are carriers of a virus or virus complex which, when introduced into Arran Victory or President, induces a top-necrosis streak. Salaman (1930, *b*) divides these carriers into two sub-groups, A and B, basing the distinction on their varietal reactions; our investigations fully support this sub-division.

Top-necrosis—Section A.—The varieties Arran Banner, Arran Consul, Green Mountain, Majestic and Up-to-Date, can carry without exhibiting any symptoms a virus complex which, when communicated by grafting to healthy President or to Arran Victory, occasions an acute top-necrosis in both varieties. A similar complex occasioning the same reaction is to be found in most samples of Duke of York and not a few of Eclipse. In these latter varieties, however, the virus is not strictly latent for they may themselves show evidence of disease, the Duke of York often developing a vague mottle and the Eclipse a more definite and blotchy one—in neither case are the plants noticeably disabled. The acronecrotic streak developed on the two test varieties is the same, whether the infection reach them from the true carrier varieties or from those which show some sign of disease themselves.

That the virus complex is the same in all is extremely probable, for we have found that reciprocal grafts between the carriers produce no reaction, which goes to prove that each of the six varieties is infected by the same virus complex, for each can carry its fellow's burden.

When Tobacco and *Datura* are inoculated with the juice from any one of these six varieties, the former reacts by a definite clearing of the finer veins of the young leaves, indicating the presence of the Y virus, whilst rings on the inoculated leaf, followed later by necrotic patches on the older ones, indicate the co-existence of the X virus. The *Daturas* react to all the sources with a severe mottle in which later green bands form alongside the veins, a reaction which is common to the X virus. On Tomato, inoculations produce a faint mottle, but if Tobacco mosaic be added to the inoculation, then a streak with leaf-drop ensues. This is the reaction which Valteau and Johnson (1931) claim to be pathognomonic for the so-called healthy potato virus, and which they find is always present in the variety Green Mountain. A stock of this



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.

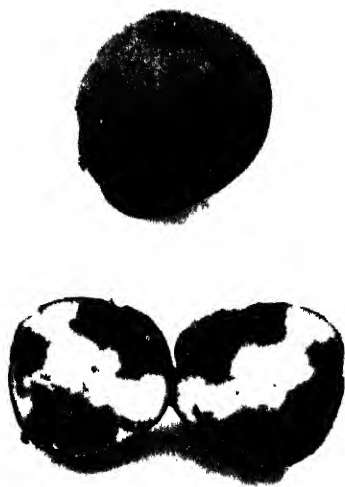


FIG. 6.



FIG. 7.

variety kindly given the senior author by Dr. Schultz as "healthy" in 1927, was found to be carrying this virus complex of top-necrosis.

Actually the presence of the Z virus has not been determined. Inasmuch as top-necrosis symptoms cannot be evoked by inoculation in any of the varieties examined (see Table I), except in Epicure and Arran Crest, we may assume that the complex responsible contains some element which renders it uninoculable. In the case of Paracrinkle, where the uninoculability is a constant feature, the presence of the uninoculable virus Z in the causative complex suggests that it is this virus which may be in some special manner responsible. It is therefore not improbable that the top-necrosis virus under consideration contains an uninoculable virus similar to the Z described, in addition to the X and Y we know are present. That the two varieties Arran Crest and Epicure react on inoculation is not surprising, for these varieties develop a top-necrosis, as we have already seen, on infection with a pure culture of the X virus. It is difficult to avoid the assumption that this sensitiveness of Arran Crest and Epicure is consequent on the ability possessed by their juices to break up the complex on contact and absorb forthwith the X. Other varieties after attempted inoculation give no evidence of the presence of any free X which we know would normally produce a mosaic in all of them; nor indeed evidence of the presence of any other recognisable virus. When sub-inoculations were made to Epicure from inoculated symptomless plants of Arran Victory, Arran Chief and President, there was no reaction in the former variety, which supports the view that the complex is not broken up, but rather that it is rejected as a whole by these varieties.

The juice of plants carrying top-necrosis A was inoculated into seedlings of certain wild potato species, viz., *S. utile*, *S. papa silvestre* and *S. antipovitchii*; all seedlings of the latter species reacted with the production of necrotic patches on the leaves, and vein-banding; it is probable that the cytoplasm of this unrelated stock disrupts the complex in much the same way as does that of Arran Crest and Epicure.

The virus complex common to the six carrier plants in which it has been found to exist in the field has been artificially communicated by grafting to a number of other varieties with the results as found in Table I.

In this table the division between those which are proved carriers and those which are probably such is purely temporary, the latter group not having been subjected to sub-culture to prove whether or not any virus is present. On the other hand, the two varieties, Duke of York and Eclipse, showing mosaic

Table I.*

	Found in the field as carriers.	Infection by graft.	Infection by needle inoculation.
Top-necrosis with no mottling—			
Arran Chief		2/2	0/2
Arran Crest		1/2	2/2
Arran Victory		6/7	0/6
Epicure		1/1	2/3
Kerr's Pink		1/2	—
King Edward		2/2	—
President		5/6	0/5
Sharpe's Express		1/1	0/2
Mosaic—no streak—			
Duke of York	+	1/1	—
Eclipse	+	1/1	—
No symptoms but proved carriers—			
Arran Banner	+	0/1	—
Arran Consul	+	0/2	—
Di Vernon		0/1	—
Green Mountain	+	—	—
Great Scot		1/2	—
King George		0/1	—
Majestic	+	0/2	—
Up-to-Date	+	2/2	—
No symptoms—probably carriers—			
Arran Comrade		0/1	—
Champion		0/2	—
Rhoderick Dhu		0/2	—
No symptoms and no infection—			
Abundance		0/4	—
Tobacco—			
27 of the 55 reactions definitely demonstrated Y as well as X		—	55/57
Datura—			
X symptoms displayed		1/1	35/48

* The fraction against each variety represents as regards the numerator the total number of plants showing a reaction, and as regards the denominator the total number of plants used.

symptoms have been proved by us to contain the same complex as the true carriers and to communicate the full top-necrosis to susceptible varieties on grafting.

We have in Table I introduced under the group-heading "No symptoms—No infection" the variety Abundance, and we have done so with hesitation.

In the course of five years' intensive study of the virus diseases in the potato, we have failed to meet any unequivocal evidence for the presence of any true resistance to any virus disease in any variety. If we cannot communicate a virus entity or complex by the needle, we can always induce an infection of

some kind by means of grafting, and apparently it is the whole complex which passes to the host by this method. A virus may be, and often is, as we have seen, carried by a variety, but that is clearly a different phenomenon to that of resistance. Possibly it is expecting too much that there should be an absolute resistance to any virus, simple or complex, but we are bound to seek for any evidence which might show that a variety may reduce the virulence of a virus (or alternatively excite it) by passage through its tissues. We have no substantial evidence for such an action, apart from that to be now adduced in respect to the variety Abundance, which in itself is by no means conclusive. On the other hand, there are several considerations and some facts besides those about to be recorded, which induce a strong suspicion that in the course of vegetative propagation from year to year some virus elements may suffer such a loss of virulence within the tissues of a plant as to be finally unrecognisable, and that under certain circumstances some degree of virulence may be once more attained.

The virus-free stocks of Abundance, *i.e.*, those stocks which have never been found to produce a reaction on our test plants Arran Victory, President, Tobacco, or Datura, have been grafted on four occasions with the top-necrosis virus, twice from Up-to-Date and once from Majestic, and once from Arran Consul carriers. In three cases there was no response whatever, although the grafts were good and grew freely. In the fourth case there was a slight clearing of the veins after 3 weeks, from which the plant entirely recovered, but at the tenth week after grafting, when both scion and stock were maturing, a few veinal necroses appeared. Scions were removed from this same plant 7 weeks after the grafting and put on healthy Arran Victory and President; in neither case did the stock plants exhibit any reaction.

The evidence is suggestive of the existence in the variety Abundance of a definite resistance to certain viruses. Salaman (1930, *b*) pointed out that this variety behaved in a peculiar manner to Crinkle A. We now know, Salaman (1932), that the virus content of the infecting crinkle was $Z + X$: on Abundance it produced but the mildest mosaic, and when returned to President it gave an interveinal mosaic and not a crinkle, which suggests that the action of the Z virus had been eliminated somehow. Datura inoculated from the infected Abundance gave a similar reaction to that evoked by the virus X, which shows that it had at any rate accepted this virus. In the same communication an account is given of the reactions of an Abundance in the field in which the leaves were a trifle rugose but in which there was no mottling; here, two consecutive passages through President reduced the reaction to vanishing point,

whilst in a similar passage through Arran Victory the plants were found to react with a mild but definite picture of Crinkle A. Here the evidence again points to an action of the Abundance on the Z virus which we may regard as attenuated in the juices of Abundance and which does not regain its vigour in the juices of President, but does do so to some extent in Arran Victory. An attempt was made to discover whether Abundance juice had any direct action on the X virus; leaves of healthy Abundance and of President plants bearing the X virus were ground up and incubated at 70° F. for 1 hour and then inoculated into plants of the following varieties—Abundance, Arran Crest, Arran Victory and President. The Arran Crest reacted promptly with a top-necrosis, as it always does to the X virus; King Edward failed to react; but President and Arran Victory developed interveinal mosaic of the same intensity as did the controls to the X virus alone. The evidence, though negative, goes to support the contention that the element which Abundance influences is not the X but the Z virus. An experiment in which a scion of X-bearing President was passed through an intermediate scion of Abundance, itself grafted to a healthy President, yielded a similar result, *i.e.*, the basal President stock responded with the normal interveinal mosaic reaction.

Top-necrosis—Section B.—Salaman (1930, *a*) drew a distinction between two groups of top-necrosis based on their reactions on the standard test varieties Arran Victory and President. Whereas in the A group both of these varieties go down with an acronecrotic streak, in this group President suffers an acute top-necrosis, Arran Victory only a rather mild veinal mosaic.

We have discovered but one variety in which this particular virus complex is latent, and that in only a few of its stocks. The variety is Di Vernon, and it was found in certain tubers collected in June, 1928, from healthy and vigorous plants in the field.

The varietal reactions of this latent virus as obtained by grafting are as follows:—No reaction of any kind was obtained by inoculating the juice of the Di Vernon source in the varieties specified in column 2 of Table II.

The group which displayed “No symptoms” in Table II must not be assumed to be carriers, for sub-grafts were made from Arran Crest, Sharpe’s Express and Great Scot to President without effect. The difficulty of making successful grafts with Di Vernon adds an element of uncertainty to any negative results. On the other hand, all Arran Victory plants which displayed a mosaic as a consequence of infection with this virus reacted on grafting to President by inducing a lethal top-necrosis.

The absence of a reaction following inoculation on any of these potato

Table II.*

	Infection by graft.	Infection by needle inoculation.
No symptoms but carried by—		
Di Vernon	1/1	—
Top-necrosis developed—		
Arran Banner	1/1	—
Arran Consul	1/1	—
Duke of York	1/1	—
Eclipse	1/1	—
Epicure	2/3	0/2
King Edward	1/3	—
Majestic	1/1	—
President	4/5	0/4
Up-to-Date	2/3	—
Mosaic—		
Abundance	2/2	—
Arran Chief (very mild)	1/1	0/2
Arran Victory	4/5	0/4
Kerr's Pink (very mild)	1/1	—
No symptoms—		
Arran Crest	2/2	0/2
British Queen	1/1	—
Great Scot	1/1	—
International Kidney	2/2	—
Sharpe's Express	1/1	—

* The fraction against each variety represents as regards the numerator the total number of plants showing a reaction, and as regards the denominator the total number of plants used.

varieties should be reviewed in the light of the reaction of this Di Vernon complex on Tobacco and Daturas.

On Tobacco we find a pure and definite Y and no sign of an X reaction. But this Y reaction is not obtained readily; in the first batch of nine tobaccos, only one reacted with clearing of the veins, an early symptom of Y infection, and then recovered. In a second batch of six plants inoculated later in the season, three reacted quite definitely with well-marked clearing of the veins and later a striking vein-banding. It is clear that this virus complex does not readily break up even in the foreign environment of a different species, such as Tobacco.

Top-necrosis A was inoculable only in the varieties Arran Crest and Epicure, both of which are highly susceptible to the X virus. Apparently a complex is rather more stable when X is absent than when it is present. In this respect one is reminded of paracrinkle, which is likewise free of any X virus and which can be conveyed neither to potato, Tobacco nor Datura by inoculation. On Datura we failed to obtain a reaction of any kind on the 35 plants inoculated, which is further evidence against the presence of the X virus. On the other

hand, if this particular stock of Di Vernon be grafted to *Datura*, we get a peculiar reaction which is identical with that described by Salaman and Le Pelley (1930) as characteristic of paracrinkle. These same *Daturas* showed no evidence of the X mottle. In view of the fact that the scions grew well, there can be no doubt that whatever virus was present entered the *Datura*, hence we may conclude that no X was present in this particular complex. Salaman (1932) has recorded how paracrinkle might be synthesised by the addition of a Y virus to the virus complex latent in this Di Vernon. Paracrinkle we know contains the Z virus, hence this Di Vernon must presumably contain it also and, as we have shown by the tobacco reaction, it certainly contains the Y virus in addition.

The Di Vernon complex under review therefore may be regarded as built up of Z and Y.

The two complexes which we have described, both of them latent in different varieties, are thus seen to differ very considerably in their reactions, and we are able to show that one, top-necrosis B, is built up of the virus elements Z and Y; the other, top-necrosis A, is built up of X and Y and that possibly another uninoculable virus such as Z is present also.

We do not wish to infer that our analysis is in any sense to be regarded as final; there may well be other virus entities involved which remain to be identified.

Attempts were made to infect a plant simultaneously with top-necrosis B and the pure X virus, *i.e.*, with the elements Z, Y and X, and thereby possibly counterfeit the complex common to the A variety of top-necrosis. When this is done on President the result is a top-necrosis, but as this may equally be due to the Z Y, of the B top-necrosis as to any newly synthesised virus complex, it is not of any immediate value in the solution of the problem. On the other hand, if we add the X virus and the B top-necrosis complex to Arran Victory, then if the X is producing any effect either on its own or by entering into closer relation with the B complex, we might expect to get a result which would be unlike the mosaic produced by the B complex and might be similar to that induced by the A top-necrosis which we know contains X and Y possibly associated with a Z-like element. This particular experiment was done twice; the B top-necrosis was introduced by a graft, the X by inoculation. In one case a crinkle was produced of a much more severe kind than that called forth by the B top-necrosis alone. In the second, the scion conveying the B complex was poor, but a severe interveinal mosaic supervened in which some definite necroses occurred such as are to be found usually in Arran Victory

infected by the A top-necroses complex. From these preliminary experiments it looks as if it might be possible to convert the B top-necrosis complex into the A complex in Arran Victory by the introduction of the X virus.

A third top-necrosis complex has been discovered in a Great Scot "wilding" potato which was kindly given the senior author by Mr. Anderson of the Board of Agriculture for Scotland in 1927, as a good example of a "wilding." In 1928 it was grafted on to Arran Victory and President without producing a reaction. In 1931 the same stock was tested out again, when it was found to present certain distinctive reactions. We suggest that this type of streak should be referred to as the C top-necrosis.

Unlike the A or B type, we have not found it in a latent condition. The Great Scot "wilding" from which the virus complex was obtained has throughout the 5 years it has been under observation appeared a sickly plant, many-stemmed, with the leaves much broadened and poorly developed, exhibiting a mild veinal mosaic on a dark-green background with a rugose surface, with edges deflected.

The outstanding characteristic of this C type is that when grafted to Arran Victory it causes a very severe or deadly top-necrosis streak with the extremely short incubation period of 9 days; on President it produces a crinkle and no necrosis—in short it reverses the reaction of the B top-necrosis. In the following table the varietal reactions of the C type of top-necrosis, so far as we have discovered them, are given.

Table III.

Symptoms.	Infection by graft.	Number of plants tested and number reacting.	Infection by inoculation.	Number of plants tested and number reacting.
Top-necrosis with no mosaic or crinkle.	Arran Victory King Edward Sharpe's Express	2/2 1/1 1/1	— — —	— — —
Top-necrosis with crinkle or mosaic.	Abundance Arran Crest	1/1 1/1	Arran Crest Epicure	2/2 2/2
Crinkle or severe mosaic with some necrosis.	—	—	Sharpe's Express	2/2
Crinkle or severe mosaic alone.	Duke of York Great Scot..... President	1/1 2/2 2/2	Arran Victory Great Scot..... President Up-to-Date	2/2 1/2 2/2 1/1
Severe mosaic with necrotic lesions.	Majestic	2/0	Majestic	2/2
No symptoms developed	Arran Chief Epicure (scion poor) Up-to-Date (scion poor)	0/1 0/1 0/1	— — —	— — —

On Tobacco the C type when communicated by needle has a peculiar reaction : at first there is a very definite local reaction of necrotic rings followed by a marked clearing of veins on which there supervenes a severe yellow necrotic etching of the veins. The leaves tend to fall and younger leaves succeed, again reproducing the clearing of veins and a fine spotty mottle. The reaction was observed in all of the nine plants inoculated. The Y reaction was most pronounced, much more so than the corresponding Y reaction of top-necrosis A. Later some of the younger leaves assume a peculiar appearance in that the lamina bulges upwards between section of the larger veins which do not seem to have kept pace with the remainder of the growing leaf.

A survey of the varietal reactions recorded in Table III shows that they are in many respects peculiar. The variety President, itself so susceptible to the top-necrosis complexes A and B, is but little affected. We have seen that, with the exception of the A type on Arran Crest and Epicure, both the A and B complexes are uninoculable. The C complex, on the contrary, is readily inoculable, though the symptoms induced thereby are not always the same as those consequent on grafting. Of particular interest is the reaction of the C top-necrosis complex on Majestic ; on four different plants we found a severe mosaic on the upper parts of the plant, accompanied by necrotic lesions which conformed to the top-necrosis type ; later veinal necroses with leaf-drop in the lower parts of the plant developed, which gave rise to a picture suggestive of an acropetal infection. It was as if two distinct diseases were acting more or less independently on the same plant.

We may point out that however distinct the exciting causes may be, the four different top-necroses in respect to their necrotic lesions differ neither in their general character, their distribution on the plant, nor histopathologically the one from the other. On the other hand, this particular complex produces in certain varieties a top-necrosis which differs from that produced by the other three, not in respect to the necrosis itself, but in the fact that the same plant exhibits mosaic symptoms which may vary from a mild mosaic to a crinkle.

Discussion.

The history of acropetal necrosis as a virus plant disease with the narrowing down of its symptoms to a series of varietal reactions, is a tale full of promise for it suggests that all the recognised virus diseases may ultimately be related to a specific virus or virus complex to which a specific range of reactions will be ascribed. Hitherto our views on virus diseases of plants have either passed, or are still in process of passing, through the phase when protean reactions

are mistaken for specific diseases and given special names. With the birth, or rather recognition, of the Y virus and its cogeners, the X and Z viruses, we may be permitted to look forward to the time when the idea that names such as mosaic, mild mosaic, rugose mosaic, crinkle, streak, and the like, connote separate disease entities, instead of mere symptoms often common to the action of a number of distinct infective agents, will be consigned to the limbo of outworn hypotheses.

In this particular instance we may be permitted to claim that a disease which has passed under very many different names, of which the more commonly known are leaf-drop streak or acropetal necrosis, is induced by one single virus, and that virus is Kenneth Smith's Y. It is further established that Quanjer's top-necrosis or acronecrosis is, both in its histological expression and in its causative agents, entirely distinct from the acropetal type. Of these causative agents it is established that in a limited number of varieties this disease may be induced by Kenneth Smith's X virus acting alone. We have learnt, however, that there are three other causative agents which will produce in one variety or another a top-necrosis; and we have distinguished them by their varietal reactions and designated them as top-necrosis A, B, and C.

We have proved that top-necrosis A contains both X and Y and we suspect the presence of Z; and that top-necrosis B contains Y and not X and that it almost certainly contains Z. We have further shown that top-necrosis C contains X and Y, but we have no particular reason to suggest that Z is present. Although the two complexes A and C do thus appear to show a like composition as far as our yet imperfect methods of analysis allow us to investigate it, we yet know that they are entirely distinct.

It has been suggested by Salaman (1932) that a virus complex is not a mere mixture but a more or less specific grouping of virus entities united in such a manner that the resultant group acts as a whole and excites reactions because of the characters which have come to it by reason of the specific grouping of its constituent units. Nothing could demonstrate this more forcibly than the fact that Up-to-Date plants carrying the top-necrosis A complex which undoubtedly contain the X and Y viruses, will succumb with a typical acropetal streak when they are further infected with the pure Y virus, a fact which would be difficult to understand were such complex to be a mere mixture. The reactions of the four acronecrotic groups would lend further support to this view of virus complexes.

Similarly, top-necrosis B contains the Z and Y virus, but so does para-crinkle, which has quite different reactions.

The problems which these facts present cannot be solved satisfactorily till we have more analytic work on virus entities ; but a solution of the problem might be expected to follow on either of the three following lines : (a) that similarity of composition, so far as the mere presence of the three viruses X, Y, and Z are concerned, does not necessarily imply a similarity in the resultant complex as shown by its reactions ; (b) that these diseases contain the same virus elements, but in different proportions ; (c) that they differ by reason of a change of virulence in one or other constituent virus.

There does seem to be an especial importance in the relation between a complex and its environment, *i.e.*, the cytoplasmic characters of the host. The fact that top-necrosis complex A will react on inoculation on Arran Crest and Epicure only of the varieties tested and on seedlings of *S. antepovitchii*, a wild tuber-bearing *Solanum*, and not at all on those of *S. utile* and only occasionally on *Papa Silvestre*, emphasises this fact. It seems reasonable to suppose that in order to exert an action, an otherwise uninoculable complex must suffer disintegration on contact with the strange cytoplasm before one or more of its constituents can act. In Tobacco this process can in a sense be observed, a complex such as top-necrosis A will exert first a local reaction due to the X virus, then a clearing of the veins due to the Y, and at this point Kenneth Smith has shown that the Y virus can be recovered without its fellow, whilst the X, free from any Y, can be recovered from the inoculated leaf. In the potato such a separation occurs but rarely.

It is not proposed to discuss the problem here in further detail. The important question of inoculability versus uninoculability, the means by which X and Z reach the potato plant at all, the possibility of viruses losing their virulence after a long residence within the same plant, and the like, must all be considered before a satisfactory solution of the nature of the potato virus complexes can be envisaged.

The study of the virus complexes concerned in the top-necroses which, as we have seen, are so readily and frequently carried by varieties such as Majestic and Up-to-Date, which are grown on a very large scale, leads to a consideration of practical and economical importance. These carriers contain the virus Y ; it is this virus which is so readily taken up and transferred by the ubiquitous aphid *Myzus persicae*. The field crops of such carriers may give the farmer every satisfaction. Looked at from the pathologist's point of view, however, they are vast reservoirs of the most destructive of all the virus entities we know, dangers to other varieties, and even a danger to themselves, for a carrier Up-to-Date can go down to a further infection of the very virus Y which it itself is carrying.

We are indeed faced by a dilemma: either we must aim at growing only virus-free stocks—a possible though difficult and costly task—or we must use only such varieties as are successful carriers of the more serious virus diseases.

Summary.

A summary of the literature on streak is given, from which it appears that two distinct clinical states can be isolated.

One of these is that described by Orton and commonly known as stipple-streak or leaf-drop streak and later designated, on the grounds of its histopathology, as acropetal necrosis. The other, known as top-necrosis, has been described by Quanjer on the basis of its histopathology as acronecrosis.

It has been shown in that the former is the distinctive reaction in certain varieties of the Y virus of Kenneth Smith.

Acronecrotic or top-necroses have been shown to be divisible into at least four distinct groups based on their varietal reaction, and here designated as top-necrosis X, top-necrosis A, top-necrosis B, and top-necrosis C.

The first three are alike in that when they do produce a top-necrosis in any given variety, it is unaccompanied by any mosaic symptom. Top-necrosis C, on the other hand, differs clinically by the fact that necrotic and mosaic symptoms occur together.

It is shown that top-necrosis X is due to the action of the X virus acting alone.

Top-necrosis A is shown to be due to a complex containing both X and Y, possibly associated with the virus Z.

Top-necrosis B is shown to be due to a complex containing both Z and Y.

Top-necrosis C is likewise shown to be due to the presence of both viruses X and Y.

The top-necroses X and C complexes are capable of transmission by needle inoculation to other potato varieties, though it by no means follows that the resultant lesion is a top-necrosis. Top-necrosis B is uninoculable, and so is top-necrosis A, except that it can be conveyed to the varieties Arran Crest and Epicure by the needle.

Carriers of top-necrosis A are found amongst many of our widest grown varieties, such as Arran Banner, Majestic, and Up-to-Date; indeed the latter is rarely to be found without such latent infection.

A carrier of top-necrosis B has only been found in the field in the variety Di Vernon.

A clinical disease, it is held, cannot be defined by the syndrome of its reaction in one particular variety of the potato, but it is to be identified rather by the complete tale of its reactions in a large number of varieties, as well as in a certain number of selected species of the non-related Solanaceæ.

The view is put forward that our goal in the study of plant virus diseases and their classification should be to find a correct formula in terms of the virus entities concerned for each clinical disease.

A discussion of the results and their bearing on the theory of virus complexes is added, certain suggestions are put forward for consideration.

The bearing of these results on the raising of crops on an economic basis is considered.

EXPLANATION OF PLATES.

PLATE 3.

- FIG. 1.—Later stages of Y infection on President: necroses are spreading along large sections of the veins and are especially noticeable on the under surface of the leaf. The intermediate and lower leaves have died and dropped, consequent on the destructive action of the virus on the supporting tissue in the petiole. The apical growth is but mildly crinkled, and free from visible necroses.
- FIG. 2.—A plant of President infected in the previous season with the Y virus. The photograph represents 3 months' growth. The plant is stunted, leaves poorly developed, twisted, and the surface mildly crinkled. There are no necroses and no leaf-drop, though both were present in the previous season.
- FIG. 3.—The earliest stage of a top-necrosis as seen in President; the necrotic flecks are scattered indiscriminately on veinal and interveinal areas and are unaccompanied by mottling or deformity.
- FIG. 4.—A President plant with advanced symptoms of top-necrosis. The upper part of the plant is already dead, the lower dying with large irregular necrotic flecks. The infecting virus complex is top-necrosis B.

PLATE 4.

- FIG. 5.—Small shrunken tubers of an infected Arran Victory plant: the tuber to left is completely converted into a corky mass; that to the right is partly so. On its outer surface deep fissures have appeared, the eyes are sunken and destroyed. The infecting virus complex is that of top-necrosis A.
- FIG. 6.—A tuber from an infected President plant which has developed during storage ulcerous fissures; on section the cork is seen to be mainly on the inner side of the vascular bundles, but extends to the periphery in the neighbourhood of the eyes, which are destroyed. The infecting virus complex is the same as in fig. 7.
- FIG. 7.—An Arran Victory plant in its second season after infection with top-necrosis derived from Up-to-Date. (Reproduced by kind permission of Dr. Kenneth Smith.)

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A Study on the Histological Changes Resulting from Certain Virus Infections of the Potato.

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[PLATES 5-7.]

Introduction.

A degeneration of the phloem elements of the potato, which occurs in plants suffering from Leaf-roll, was first described by Quanjer (1913). Since then phloem necrosis has been recorded by many workers, some ascribing it to specific pathogenic causes while others state that it occurs in both healthy and diseased plants. Esmarch (1921) states that it is always present in the ripening plant, and v. Brehmer (1923) shows three types of necrosis, viz., *Nekrobiose*, *Obliteration* and *Necrosis*, of which only the last is necessarily due to virus infection. On the other hand, Artschwager (1923) found necrotic changes only in virus infected and injured plants.

In this paper an attempt is made to correlate the histopathological changes occurring in virus diseased plants with the external symptoms which they exhibit. A large part of the work is devoted to the morbid anatomy of certain "Streak" diseases, for a detailed account of the external symptoms of which see Salaman and Bawden (1932).

All specimens examined were taken from plants grown under glass. They were fixed in chrome-acetic acid fixative, dehydrated and embedded in the usual manner. Sections were cut at a thickness of 10 μ . The two stains found to be most effective for differentiating necrotic from normal tissue were Safranin and Pianese III B (Conn, 1929). For rapid work the latter was especially good, 20 minutes in the stain being sufficient to differentiate between healthy tissue and that showing even the earliest pathological changes.

Healthy Stem.

For controls, specimens were taken from believed virus-free plants of Arran Victory and President, kept under observation and grown under insect-proof conditions for 6 years, and for purposes of comparison a short account of a cross section of such a plant is here given.

The stem is bounded on the outside by a single epidermal layer, beneath which is another single layer of rather regular cells, the sub-epidermal layer. Within this is a well-marked collenchyma of three to five cells in thickness. These cells are considerably thickened, more especially at their corners. Inside the collenchyma are one or two layers of parenchymatous cells which are limited on the inside by a rather ill-defined endodermis. Within this is the pericycle and then the bicollateral bundles. The wood is separated from the outer phloem by the cambium, and from the inner phloem by parenchymatous cells, the perimedullary zone. The outer phloem bundles become linked up by the formation of secondary phloem and the wood bundles join by the formation of secondary interfascicular wood, so giving rise to a continuous vascular cylinder. The internal phloem groups in cross section always appear as isolated elements. The wood is made up of vessels, tracheids and wood parenchyma, and the phloem of sieve tubes and companion cells. The centre of the stem consists of large round and fairly regular pith cells.

In the mature stem there is a considerable amount of secondary phloem, but the primary elements remain distinct and normal. The walls of the sieve tubes sometimes become slightly thickened with cellulose but show no pathological changes. No sign of the *Nekrobiose* and *Obliteration* described by v. Brehmer (1923) were seen in any stem of the virus-free plants examined. It should perhaps be emphasised here that the plants were grown under glass.

In this work the division of the main Streak diseases of the potato into Acronecrosis and Acropetal necrosis as laid down by Quanjer (1931) has been accepted and found to rest on very real differences. The acronecrotic group has, however, been found to be readily divisible into subgroups, all differentiated by their varietal reactions. In this investigation, numbers of these subgroups have been subjected to histological examination and no difference observed between them. The writer is therefore able to endorse Quanjer's division of the Streak diseases into two main groupings which can be readily distinguished by their morbid anatomy. The terms *Acronecrosis* or *Top-necrosis*, and *Acropetal necrosis* or *Leaf Drop Streak*, as employed by Quanjer, are used throughout.

Acronecrosis (Top-necrosis).

The first external symptoms of Top-necrosis are an extensive necrotic spotting of the uppermost leaves, and are followed by the dying of the plant from the top downwards. The absence of any mottling is characteristic of this expression of disease. Microscopical examination of stems of plants showing these external symptoms in all cases reveals the presence of necrotic areas, fig. 1,

Plate 5. These are more severe in the distal portions and become much less pronounced towards the base of the plant. This corresponds to a similar decrease in severity of the external symptoms towards the base of the plant. Several varieties of plants showing Top-necrosis were examined and the internal symptoms in all cases were identical; thus a general description of them is deemed sufficient.

The necroses arise in the phloem, usually, but by no means invariably, in the internal phloem, and from this they spread into other neighbouring tissues. The first pathological symptom is the thickening of the cell walls of the primary phloem elements. This thickening is accompanied by the separation of adjacent primary cell walls. Thus intercellular spaces are formed and these are filled with a yellowish brown gum-like deposit. The necrotic process passes into the neighbouring parenchyma, and it is in this, owing to the greater size of the cells, that the formation of intercellular spaces is most pronounced, fig. 2, Plate 5. The changes, however, appear similar in both phloem and parenchyma. The thickening of the walls and the formation of intercellular spaces are frequently so advanced as to cause the complete obliteration of the sieve tubes and the collapse of the companion cells. Changes also occur in the cell protoplasm. This may disappear altogether during the thickening of the wall, or it may turn into a plastic mass similar in appearance and reaction to that found in the intercellular spaces. Adjacent cells in the same necrotic area frequently exhibit these different changes, the one being thick walled and empty and the other filled with a darkly staining deposit.

The necrosis spreads from the internal phloem in all directions, but most markedly through the perimedullary zone towards the wood. The wood parenchyma is particularly susceptible. These cells become severely discoloured, many collapse completely, and others become filled with a plastic deposit similar to that mentioned above. In severe cases the xylem vessels are involved. Layers of deeply staining material are laid on the walls and the whole vessel may become filled with gum, fig. 3, Plate 5. Occasionally tyloses are produced and the vessels are blocked by these.

Necrotic changes originate with less frequency in the outer phloem. Such changes, however, do take place and are essentially similar to those already described, with one exception, fig. 6, Plate 6. The main spread of the necrosis from the outer phloem is centripetal and not centrifugal, i.e., its main spread is also towards the wood. An interesting point is that usually in any one section examined only a small number of the phloem groups are necrotic; large necrotic areas may be seen radiating from one or two phloem groups whilst

the remainder are perfectly normal, fig. 1, Plate 5. Artschwager (1918) has shown that all the phloem groups anastomose freely and it is difficult to understand why of two groups at the same level one should become necrotic and not the other.

At the distal portion of the stem the necroses penetrate through the vascular bundles into the pericycle and cortex, thereby leading to the death of all the tissues, fig. 4, Plate 5. In old plants, more especially when grown at high temperatures, this killing out is confined to the tender growing point, the spread of the necroses lower down the plant being restricted. Such plants on examination show the production of cork cambiums around the necrotic areas, and this serves partially to localise the effects of the disease, fig. 5, Plate 6. This formation of cork cells may account for the slow rate of spread, sometimes indeed the complete cessation of spread, not infrequently experienced when infected plants are kept at high temperatures. In young tender plants such phellogens are apparently not formed and the first symptoms at the apex are followed by the death of the whole stem.

Necroses are also found in the petioles, which in their origin and appearance are identical with those in the main stem. Frequently, however, they are particularly severe, all the phloem groups being involved, and the whole vascular tissue degenerate. In such cases the leaf of necessity shrivels and dies. The axillary buds also become affected, necroses arise in the young vascular bundles and spread from these, ultimately killing the bud.

From the staining reactions given by the necroses, it appears that the walls of affected cells are suberised whilst the deposits in the cells and intercellular spaces are of a gummy nature, rich in pectin. With Safranin differential staining is obtained, viz., dark red in cell walls and orange red in cell contents. A red colour is also obtained with Sudan III, ammoniated Fuchsin and Alkanin, and a brownish red with Ruthenium Red. With Iodine Green the walls become green and the cell contents a bluish green. The necroses are free from lignin for they give no reaction with Phloroglucin and HCl. They are insoluble in concentrated H_2SO_4 , boiling Schulze's solution and alkalis, the last merely intensifying the natural colouring of the necroses. After 4 to 5 hours in Eau de Javelle they are dissolved. The natural deep yellow colour of the degenerate areas renders specific tests difficult and many stains useless.

The leaves of affected plants exhibit severe pathological changes showing externally as black spots which penetrate the thickness of the leaf. The point of origin of these necroses has not been definitely ascertained, but in each necrotic area examined, however small, degenerate vascular bundles were

found, and it is believed that the necrotic process begins in these, most probably in the phloem elements of the minute bundles. The leaf parenchyma cells lose their contents, become suberised and collapse. Prior to the process of suberisation the cells swell, their protoplasm becomes granular and stains deeply, while the plastids degenerate and give rise to a glutinous mass, remains of which may be seen on the dead cell walls.

No intracellular or "x" bodies were seen in cells of plants showing symptoms of Top-necrosis.

Tubers obtained from Top-necrotic plants are usually of two kinds, some normal and others showing pathological changes, visible to the naked eye in the cut tuber as darkly coloured areas in the parenchyma. When examined microscopically the necroses are seen to develop in essentially the same manner as those found in the stem, the first symptom being a yellowing and thickening of the walls of the phloem groups. The necrosis spreads rapidly to the storage parenchyma, and the phloem bundles collapse, fig. 7, Plate 6. The contents of the cells disappear, and the walls are suberised. The necrotic areas are surrounded often incompletely by moderately developed phellogens which cut off regular layers of cork cells. The interior of a large necrosis thus becomes a mass of hard corky cells, fig. 8, Plate 6. Starch grains, although abundant in other parts of the tuber, are absent from the otherwise normal cells abutting on the cork cambiums. In cells slightly further from the latter starch grains in all stages of dissolution may be seen. In the tuber, as in the stem, in the early stages usually only a small number of the phloem elements seen in any one section are necrotic. Thus the tuber reacts to the disease by producing cork cambiums, in the formation of which the starch is used up. Rarely, however, are the phellogens sufficient to stay the progress of the necrosis, and tubers examined after a few months' storage are frequently reduced to a mass of cork. The necroses spread into the eyes of the tuber and cause their death.

The symptoms described above have been noted in many varieties of potato when infected with apparently different viruses or virus complexes. Plants in which necroses were found, together with the source of the virus, or virus complex, producing these changes and the method of infection are shown in the following table.

All the plants in the table revealed the external symptoms of Top-necrosis. Infected plants which did not show these external symptoms exhibited no necroses radiating from the phloem groups, *e.g.*, Arran Victory plants grafted with the Di Vernon carrier, which causes a necrosis in many varieties, itself exhibits the external symptoms of a mosaic, and the tissues of such plants

Table.

Variety.	Source of infection.	Method of infection.
President	Up-to-Date carrier	Grafting.
"	Di Vernon carrier	"
Arran Victory	Up-to-Date carrier	"
Majestic	Di Vernon carrier	"
Eclipse	"	"
Duke of York	"	"
Arran Chief	Up-to-Date carrier	"
Sharpe's Express	"	"
Kerr's Pink	"	"
Epicure	"	"
"	"	Needle inoculation.
"	President Crinkle A.	Grafting.
"	"	Needle inoculation.
"	Di Vernon carrier	Grafting.
Arran Crest	Up-to-Date carrier	"
"	"	Needle inoculation.
"	President Crinkle A.	Grafting.
"	"	Needle inoculation.
King Edward	Up-to-Date carrier	Grafting.
"	President Crinkle A.	"
"	"	Needle inoculation.

are found to be normal. It was further found that stems of such carriers as produce Top-necrosis in other varieties, *e.g.*, Up-to-Date, are also normal. Thus the formation of these necroses is not dependent solely on the presence of any given virus, but has a very definite relation to the internal environment, *i.e.*, to the varietal characteristics of the plant juices of the host plants.

Acropetal Necrosis (Leaf Drop Streak).

The external symptoms of Acropetal necrosis are a crinkling of the uppermost leaves and a necrosis which causes the dropping of the lower leaves, which remain hanging on to the main stem as if by a thread. Microscopical examination of stems of plants showing such symptoms may or may not reveal internal necrotic areas, depending greatly at which part of the stem the sections are taken. Those taken at the distal portion rarely, if ever, show any, and sections from the centres of the internodal areas only do so in the late stages of severe attack. On the other hand, severe necrotic changes are almost always found just below the nodes at which a leaf has fallen.

These necroses in unstained sections appear yellowish brown and, affecting chiefly the collenchyma, are confined to the cortex. Occasionally the epidermal and subepidermal cells are also involved. The "wing" also is frequently necrotic. The vascular bundles of such plants are normal, fig. 9, Plate 7.

The degenerative changes occurring seem to be similar to those in Top-necrosis. The cell wall becomes suberised whilst the cell contents either disappear (when the cell may collapse), or the contents are changed into a gummy mass which fills the lumen. The formation of intercellular spaces, however, is not so pronounced. Another difference is in the direction of spread of the necrosis. In Acropetal necrosis this is mainly perpendicular, and not, as in the Acronecrotic type, mainly towards the wood; it thus gives rise to dark superficial stripes, visible under the epidermis, which may itself remain green. The necrosis further spreads around the stem along the collenchyma forming narrow arc-like stripes as seen in transverse section, fig. 9, Plate 7.

The necrotic changes in the petiole are similar in character but usually much more severe; frequently the whole cortex is changed to a corky mass, and the degeneration may penetrate through the parenchyma separating the vascular bundles to the pith, fig. 10, Plate 7. After the fall of the leaf the whole petiole shrivels and dies leaving only the wood vessels unaffected. The vascular system of axillary buds is normal and, although the scale leaves may develop extensive lesions, the buds are not killed and retain their power of growth.

It would appear that it is in the leaf that the necrotic changes first occur, and from thence the spread extends along the petiole and into the main stem. Changes are first observed in the leaf parenchyma cells surrounding the veins. The walls of these cells swell up, become discoloured and take on the staining reactions of cork and cutin, fig. 11, Plate 7. Later the plastids degenerate and the contents of the cell disappear leaving a thick walled empty dead cell. These frequently collapse leaving a debris of dead cells surrounding an apparently normal vascular bundle. The necrosis spreads from the vein through the leaf tissue, and more rapidly along the collenchyma of the petiole. The presence of this dead tissue renders the petiole brittle and it falls under its own weight, but remains hanging to the main stem by means of the unaffected wood vessels. The absence in Top-necrosis of the picture of the hanging leaf, so characteristic of Acropetal necrosis, appears to be due to the fact that in the former the necrosis is chiefly of the vascular tissues, whilst in the latter it affects the supporting tissues only.

No intracellular or "x" bodies were found in cells of plants showing symptoms of acropetal necrosis. In leaves of President plants showing this disease curious circular sac-like deposits of crystals were found, fig. 12, Plate 7. These were of considerable size and occurred in the same leaf in the completely necrotic tissue and in that still otherwise normal. They occupied large intercellular spaces, in some cases filling the whole thickness of the leaf. It is

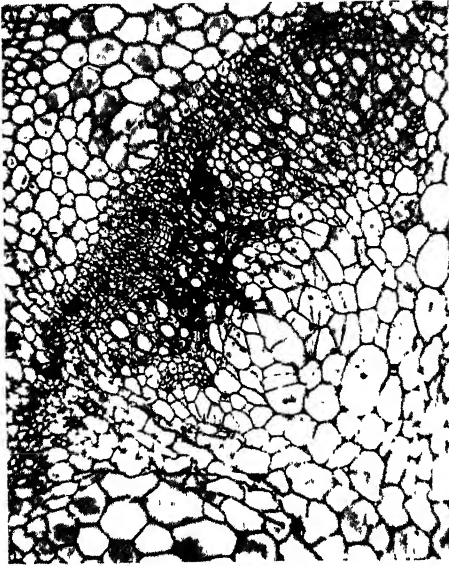


FIG. 1.

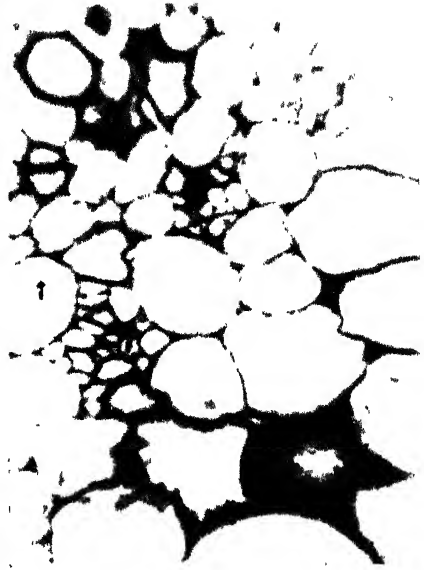


FIG. 2.

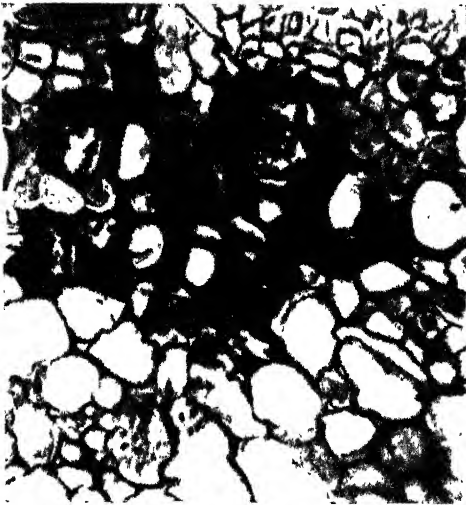


FIG. 3.



FIG. 4.

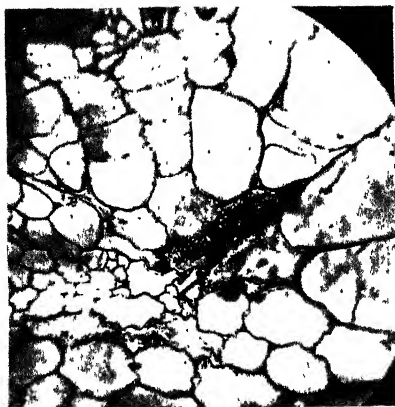


FIG. 5.

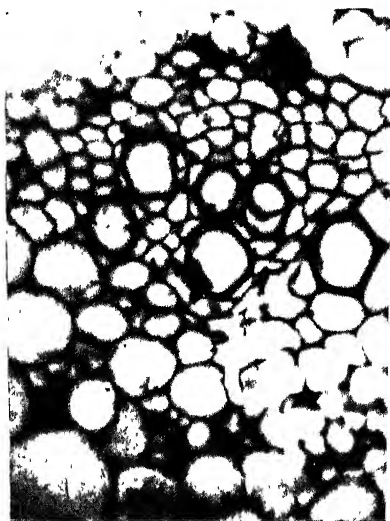


FIG. 6.



FIG. 7.



FIG. 8.

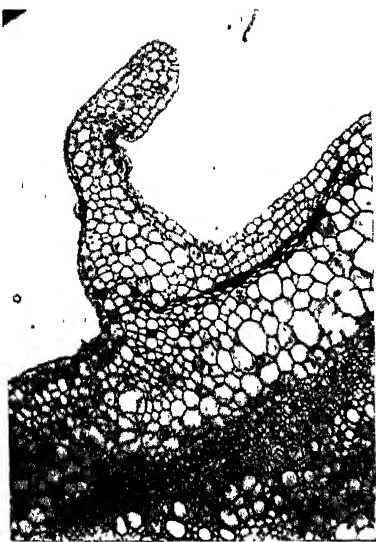


FIG. 9.

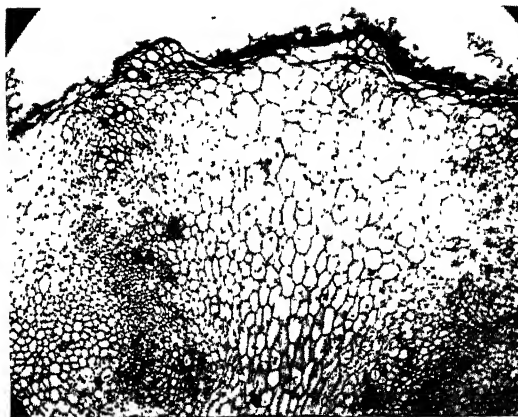


FIG. 10.

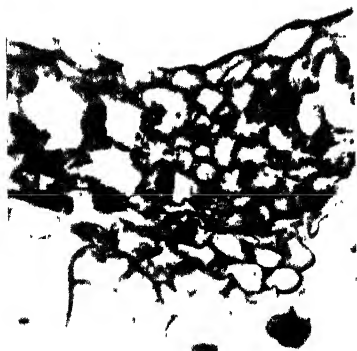


FIG. 11.



FIG. 12.

not known if these have any special significance or are connected in any way with the virus, but they were not found in any healthy plants.

Symptoms of Acropetal necrosis as yet have been produced by only one virus, viz., Kenneth Smith's "y" virus (1931). Internal changes of the type described above have been seen in President, Up-to-Date and Majestic. All these varieties show the typical leaf drop streak. Stems and petioles from plants of Arran Victory and Epicure infected with the "y" virus were examined microscopically and their tissues were found to be normal and free from necroses. These varieties react to the virus by showing symptoms of a crinkle and not leaf drop streak. Thus the internal and external symptoms of infected plants are closely correlated, and the production of internal necroses in the presence of the "y" virus is essentially a varietal reaction.

Leaf Roll (Phloem Necrosis).

In order to compare the different types of necrosis occurring in potato plants, specimens of stems and petioles were taken and examined from six President plants showing typical symptoms of Leaf roll. Two of these were infected with the disease in the current season and the phloem of these was normal. The others were progeny of plants infected in previous years, and of these one was also normal whilst the others showed necrotic phloem elements. The necrotic process consists of a thickening of the walls of the primary phloem groups together with a slight separation of primary cell walls, so giving rise to small intracellular spaces. Occasionally this is sufficiently severe to cause the obliteration of the sieve tubes. The process appears to be one of lignification, for the affected phloem groups give a red colour when acted upon by Phloroglucin and HCl. The necroses occur equally in the internal and external phloem, but in the latter they are usually limited to the primary elements. No spread of the necrosis was seen to the neighbouring tissues. In the three plants similar changes were found in the petiole.

The necrotic changes occurring in relation to Leaf roll thus differ from those of Top necrosis in the following ways: chemically; locally, in that they are restricted to the phloem, and temporally, in that they are not always produced in the first season. This is in fair agreement with the findings of Quanjer (1931) except that he describes a very faint necrosis in the first season. It is difficult to be sure what exactly are Artschwager's conclusions on this subject, for in one paper he states: "The anatomical studies of both European and American Leaf roll have so far failed to show a distinct correlation with the

external symptoms exhibited by the plant," and "in no instance could the presence of lignin be shown" (1918), whilst the same worker later states, ". . . obliteration of the phloem is always observed in connection with Leaf roll," and "these changes consist in lignification" (1923). It is possible that these contradictory statements are due to the fact that in his first work Artschwager was dealing with plants, some infected in the current season, and others in previous seasons, whilst in his later work he was dealing only with plants infected in previous seasons. As this worker also describes a necrotic process of cutinisation spreading from the phloem to other tissues, it would seem doubtful whether he worked with plants infected with Leaf roll alone.

Leaf roll plants which show necrotic phloem usually have only a small proportion of the whole phloem affected, and the writer finds the same difficulty as did Murphy (1923) in believing, with Quanjer, that the rolling of the leaves is brought about by the presence of this necrotic conducting tissue. Indeed, as the rolling of the leaves is frequently pronounced in the first year of infection and phloem necrosis either absent or very faint, it would seem more probable that the pathological conditions of the leaf lead to the necrosis of the phloem.

Discussion.

The great variety of symptoms produced by any given potato virus or virus complex when present in different varieties has always been a feature of these infections. The result obtained by infecting a potato plant with a virus disease depends quite as much on the variety of the plant infected as on the virus used. Thus the virus causing a leaf drop streak (Acropetal necrosis) in President produces a mild crinkle in Arran Victory, whilst that causing a lethal top necrosis streak in President produces a still more mild crinkle in Arran Victory. Corresponding to this varietal expression of any given virus is the histo-pathological picture induced: where in the infected plant external necrotic change occurs, be it of the acropetal or acronecrotic type, so in the tissues will be found the corresponding histological characters, a necrosis of the supporting collenchyma in the one case, that of the phloem groups in the other. When in the plant the virus produces only a crinkle or mosaic, so in the tissues of the stem and petiole no necroses are to be found.

Quanjer (1931) has brought forward a scheme of classification of potato viruses based on the morbid anatomy of infected plants. The external symptoms, especially of leaf roll and acropetal necrosis, are, however, much more definite and recognised with much greater ease than are the internal

changes, so that the writer finds it difficult to understand what advantage is gained by using the latter as criteria for classification rather than the former. Quanjér states that this method of classification will "help to clear the ever increasing mosaic group." He might almost have stated that it will cause the complete disappearance of the mosaic group, for necrotic symptoms can apparently be produced by nearly all potato viruses if only the right variety of potato be infected with them. With the exception of those caused by the "y" virus these symptoms seem to be of the top necrotic type, and thus such a classification would merely give rise to a top necrosis group as large and chaotic as the present mosaic group.

Apart from their use as criteria for classification, these histo-pathological changes offer a promising field for research on the pathogenicity of viruses. Further work is needed to explain why the virus causing Acropetal necrosis should attack the collenchyma in particular, and those causing Leaf roll and Acronecrosis the phloem. It may be that these are the tissues in which the different viruses normally travel. It is improbable that the specificity of first attack is due solely to specific enzyme action, for once the necrotic process of Top necrosis has originated in the phloem it can then proceed to attack all tissues. The differences between the necroses of Leaf roll and Top necrosis may be explained by assuming that in the latter the action of the disease is directly on the tissues, while in the former the necrosis is a secondary process, resulting from an upset in the metabolism.

Summary.

Evidence is presented showing the occurrence of three distinct types of necrosis in virus infected potato plants, each type being correlated with a definite set of external symptoms. The origin, spread and composition of these necroses is described.

(1) *Acronecrosis*.—The external symptoms are a necrotic spotting of the uppermost leaves, followed by the dying of the plant from the top downwards. Internal symptoms are produced in the petioles, stems and tubers, and consist of necrotic changes which originate in the phloem, and spread from thence to all other tissues. In the tuber always, and occasionally in stems grown at high temperatures, phellogens are formed around the necrotic areas.

(2) *Acropetal necrosis*.—The external symptoms are a crinkling of the upper leaves, and a necrosis and falling of the lower leaves, which, however, remain hanging to the stem. Internal symptoms are seen in the stem and petioles,

and consist of a necrosis affecting chiefly the collenchyma, the vascular tissues being normal.

(3) *Leaf roll*.—Necroses are produced in the phloem of plants suffering from Leaf roll in the year following that of infection. The necroses are restricted to the phloem elements, and consist in lignification.

No necroses were found in stems or petioles of virus-free plants.

The writer's thanks are due to Dr. R. N. Salaman for the help and advice which he has freely given throughout the work, and for permission to make use of the material which forms the subject matter of this communication.

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EXPLANATION OF PLATES.

Figs. 1 to 8 are photo-micrographs of sections taken from plants showing symptoms of Acronecrosis (*Top-necrosis*).

PLATE 5.

- FIG. 1.—Section of stem showing a large necrosis in the vascular tissue. This has spread from a few degenerate phloem groups. The majority of the phloem is normal. $\times 75$.
 FIG. 2.—Section of the stem showing the origin and spread of the necroses. Two phloem groups are affected, intercellular spaces are formed and some of the sieve tubes obliterated. Note the different effect on adjacent parenchyma cells: one is thick-walled and empty, the other filled with a dark gum-like mass. $\times 340$.
 FIG. 3.—Section of stem showing advanced necrosis in the xylem. The wood parenchyma is obliterated, and the vessels are more or less filled with gum. $\times 340$.
 FIG. 4.—Longitudinal section through the apex of the stem. The uppermost leaves are completely necrotic, and within the stem all the tissues are involved. $\times 25$.

PLATE 6.

- FIG. 5.—Section of the stem of a plant grown at high temperatures. A phellogen forming around the necrosis has cut off regular layers of cork cells. $\times 340$.
- FIG. 6.—Section of stem showing necroses appearing in both the internal and external phloem. $\times 340$.
- FIG. 7.—A transverse section of the tuber showing a necrotic phloem group, and the necrosis spreading from it to the neighbouring parenchyma. $\times 340$.
- FIG. 8.—Section of a tuber showing a large necrosis. The centre is a mass of cork around which is seen regular layers of cork cells produced by the phellogen. The cells abutting on this are devoid of starch. $\times 75$.

PLATE 7.

Figs. 9 to 12 are photo-micrographs of sections taken from plants showing symptoms of Acropetal necrosis (Leaf Drop Streak).

- FIG. 9.—Section of stem showing a strip-like necrosis in the collenchyma, the majority of which has collapsed. The "wing" is also involved. $\times 75$. Note the vascular tissues in figs. 9 and 10 are normal.
- FIG. 10.—Section through a node exhibiting severe necrosis. The whole cortex of the petiole is necrotic and the pith is slightly involved. The main vascular tissues of both stem and petiole are normal. Note normal xylem vessels by which the fallen leaf remains attached to the stem. $\times 75$.
- FIG. 11.—Transverse section of a leaf showing the beginning of the necrotic process. The walls of the cells around the vein are considerably swollen and thickened, and take the staining reactions of cork and cutin. $\times 340$.
- FIG. 12.—Transverse section of a leaf of the variety President showing the presence of a large "sac-like" deposit of crystals in the necrotic tissue. $\times 340$.
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Experiments on Growth and Inhibition. Part III—Inhibition and Growth Promotion.

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1. *Introduction.*

In a Leguminous seedling, and probably in most young Dicotyledons, the rapidly growing leaves near the apex of the shoot inhibit the growth of axillary buds (except of those that are very close beneath them), and they also tend to inhibit the growth of other shoots, if any are present, and subsequently to kill them. But at the same time they promote the elongation of their own shoot below them, and also stimulate its growth in thickness. The question therefore arises how it is that the growing leaves tend to affect other shoots in the opposite way to that in which they affect their own shoot. This question was brought to notice very clearly by experiments previously reported (SNOW 1931, *b*). For it was found that in a young pea or broad bean plant that possessed two equal shoots springing from the same level, if one of the shoots was deprived of its growing leaves, it was quickly inhibited in growth by the other shoot, and was killed after about 4 weeks. The question therefore arose how it was that the defoliated shoots were inhibited and killed by the intact shoots, of which the rapidly growing leaves are the inhibiting members, whereas the growth of the intact shoots themselves was not inhibited, but increased, by their own growing leaves above them.

It was made clear that the explanation must be that the defoliated shoots were differently situated in relation to the growing leaves of the intact shoots; and it was pointed out that they were differently situated in that, firstly, any influence entering them from these leaves must be travelling upwards in them instead of downwards, and, secondly, they were out of the line between the growing leaves and the roots. But there was also a third difference not mentioned previously, for the stimulus for cambial growth, proceeding from the growing leaves of the intact shoots, travelled down those shoots, but did not spread up into the defoliated shoots, since it is unable to travel in the morphologically upward direction.

In order to explain this last point, it is necessary to recall the fundamental

investigations of Jost (1893), who showed that as a general rule (though there are exceptions) cambial growth in stems only takes place under the influence of growing leaves, and further that their influence travels only downwards. He also showed that in *Phaseolus* the leaves exert their influence on cambial growth even in seedlings grown continuously in darkness. It has been suggested that this influence—the “cambial stimulus” as it will be called—may be a hormone (Kastens, 1924), but the question of its nature will here be left open.

To return to the three points mentioned above concerning the situation of the defoliated shoots, the purpose of the following experiments was to determine which was the critical factor which led to their being inhibited and killed by the growing leaves of the intact shoots. Some further questions of interest arose incidentally.

2. The Effects of Different Orientations.

The purpose of the first experiment was to investigate the first of the three factors mentioned above by determining whether the inhibition of a stem or portion of stem depends on the way in which it is oriented with regard to the influence reaching it from the growing leaves. It was carried out as follows.

Experiment 1.—Young seedlings of *Vicia Faba* were decapitated in the epicotyl, as before, and when the axillaries of the cotyledons had grown out, those plants were selected in which they were not very unequal. The slightly larger of the two axillary shoots of each plant was defoliated, from below

upwards, until its largest remaining leaf was less than 1 mm. long, and then one of its young internodes was split throughout its length by a cut made in a median longitudinal plane with a very fine scalpel (see fig. 1). The cut was continued downwards just through the node below, and then turned to one side and passed out to the surface, as shown in the figure, so that one half of the split internode was left as a downward-pointing strip joined to the rest of the plant at the top only. This half will be called the “farther” half, and the other the

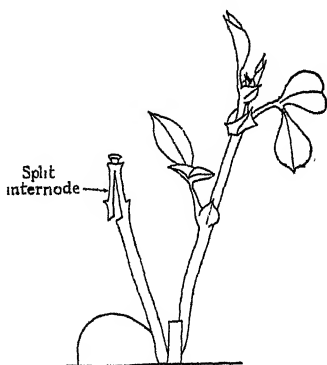


FIG. 1.

“nearer” half, since it was nearer to the rest of the plant. It was now necessary to compare the elongation of the two split half internodes, and thus to try to discover whether the farther half, in which any influence coming

from the growing leaves of the other shoot must travel downwards, would be inhibited as strongly as the nearer half.

The results obtained with six plants are given in Table I. In the first three plants the leaves less than 1 mm. long were allowed to remain on the defoliated shoots, but in the second three these leaves were also removed, together with the stem apex, by a cut passing very close behind the apex. In all except the first two plants, the splits were made in the plane of the leaves, which is the plane of symmetry of the shoot, the phyllotaxis being distichous.

Table I.

Number of plant.	Length of split internode at start, in millimetres.	Length of nearer split half of internode after 11-14 days, when fully-grown.	Length of farther split half of internode after same times.
1	4.5	9.5	11.0
2	3.0	7.0	5.5
3	4.5	7.0	7.0
4	2.25	3.75	3.75
5	5.0	6.5	6.5
6	7.0	10.0	10.0

The table shows that the half internodes of the defoliated shoots grew almost equally, but much less than the corresponding internodes of the intact shoots, which reached lengths of from 20 to 30 mm. Admittedly the diminution in their growth must have been partly due to a direct effect of the split: for it was found that even in normal single shoots splitting does diminish the growth of a young internode, though not nearly so much as it was diminished in the above experiment. Nevertheless, the growth of the nearer half internodes must also have been diminished to some extent by the inhibiting effect exerted upon them by the intact shoots. For firstly the experiments of the previous paper showed that in "two-shoot" plants of this kind, a shoot defoliated in this way is regularly inhibited by the other shoot, and secondly it was clear that the defoliated shoots were actually inhibited; for the next internode above the split grew only a few millimetres and then stopped. Since therefore the nearer halves must have been inhibited to some extent, and since the growth of the farther halves was about the same, it follows that the latter must also have been inhibited to about the same extent. It might indeed be objected that the farther halves were checked in growth not because they were inhibited, but merely because the tissues connecting them with the rest of the

shoot were not sufficient to supply them with nutriment. But this was not so : for experiments to be reported in Section 4 will show that in shoots that are not inhibited, similar downward-pointing strips, cut out from internodes of the same length or even less, grow on rapidly.

It therefore follows that the way in which a portion of stem is oriented with regard to the influence reaching it from the growing leaves, cannot be the critical factor which determines whether it shall be inhibited or not. For in the farther halves of the defoliated shoots, any influence coming from the growing leaves of the intact shoots must have been travelling in the morphologically downward direction, just as in the intact shoots themselves. This conclusion will be supported by observations made during another experiment, to be reported in Section 6, which show that downward-pointing strips cut out in the same way from mature parts of shoots exposed to inhibition, are killed quite as quickly as the other parts of those shoots. It must be specially noted that the stimulus for cambial growth coming from the growing leaves of the intact shoots, could not penetrate into the defoliated shoots at all, since it never travels upwards from one shoot into another. This rule is strictly followed in *Vicia Faba* as in other plants, as will be shown later. Consequently it was possible with the above arrangement to study the effects of different orientations without the complication of the cambial stimulus.

3. *The Effect of the Cambial Stimulus.*

Since it is now clear that the orientation of a portion of stem does not *by itself* make any difference to its susceptibility to inhibition, it is necessary to determine which of the remaining two factors mentioned in the introduction is the critical one. That is to say, it must be ascertained whether in the plants with two shoots the defoliated shoots were inhibited and killed because the cambial stimulus (or some other growth-promoting influence) could not enter them, or because they were out of the line between growing leaves and roots. For this purpose it is necessary to determine whether a length of mature stem that receives the cambial stimulus is thereby protected from being killed, even if it is out of the line between growing leaves and roots, and also whether a similarly situated portion of young stem is protected from being inhibited in its elongation. The former question can be readily answered, for it has often been observed, for instance by Jost (1893), that downward-pointing strips cut out from the lower parts of growing shoots and attached at the top only, continue to grow actively in thickness, whereas

similar upward-pointing strips, attached at the base, do not grow. Clearly therefore the downward-pointing strips are not likely to be killed, although they are out of the line between growing leaves and roots. But it seemed desirable to repeat the experiment on *Vicia Faba*, and to record briefly the processes of regeneration that take place, for comparison with the results of later experiments. Accordingly it was carried out as follows:—

Experiment 2.—Seedlings of *Vicia Faba* were grown until they had expanded their third or fourth leaves. Then a median split was made in one of the fully-grown internodes, and one of the half stems so formed was cut through either at the base of the split (in seven plants) or at the top of it (in four plants),

so that either a downward or an upward-pointing strip was formed (fig. 2). The strips were from 30 to 45 mm. long. The cut surfaces were vaselined, and above the splits the shoots continued to grow strongly.

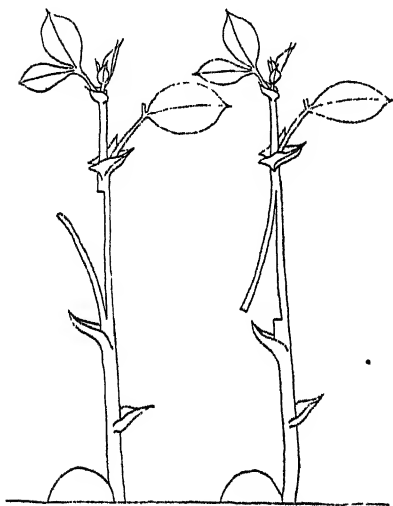


FIG. 2.

It was found that in the seven plants with downward-pointing strips, the strips were still perfectly healthy after from 43 to 53 days (in a heated green-house, but in winter). They were then examined microscopically, and it was found that the original semicircles of cambium in them had continued to grow actively, and had formed approximately the same amounts of xylem and phloem as had

the cambia in the other halves of the same stems. Also in both halves the well-known process of regeneration, by which parts of rings of cambium or procambium gradually form complete rings again, was about equally advanced. For a new cambium was beginning to spread across from the cut edges of the old cambium through a region of new tissue formed by regeneration in the pith, beneath the cut surface.

On the other hand, in three of the four plants with upward-pointing strips, after from 32 to 42 days in the same conditions, the strips had turned yellow and died from their tips to about half-way down. In the fourth plant, after 52 days, the strip had died from the tip to more than half-way down. At the end of these periods the lower parts of the strips were examined microscopically and it was found that in two of them the cambia had scarcely grown,

if at all, and scarcely any regeneration had taken place at the cut surfaces. In the other two also the normal growth of the cambium had scarcely continued, but a little regeneration had taken place at the cut surfaces, though very much less than in the downward-pointing strips. Small patches of new tissue, however, had been formed by divisions at the cut ends of the semicircle of cambium, and in one of the strips (the one that had lived the longest) the pith cells beneath the cut surface had enlarged and divided a few times parallel to the surface. There was no new cambium spreading through the pith. The regeneration in these two strips was very much less than the regeneration in the downward-pointing strips or in the other halves of the same plants, and consequently it is clear that in the main the regeneration beneath the cut surface is activated in the same way as the normal growth of the cambium—that is, by the influence transmitted downwards from the growing leaves.*

The results reported above show that in *Vicia Faba*, as in other plants, parts of stem that receive the cambial stimulus survive and grow in thickness, even if they are out of the line between growing leaves and roots, whereas parts that do not receive it, if they are out of this line, slowly die. It therefore seems that the cambial stimulus protects the parts which it reaches from being killed. It is, however, theoretically possible that it is not really the cambial stimulus itself which protects these parts, but some other unknown influence coming from the growing leaves, and travelling according to the same rule—that is, downwards only. An essentially similar opinion seems to be implied in some remarks made recently by Priestley (1930, p. 330) about a phenomenon well known in the pruning of woody shoots. For he points out that if a stub of pruned stem is left above a node, cambial activity will spread downwards from the bud at the node when it grows, and there will be no renewal of cambial activity in the stub above the node, which will wither and dry, or decay.

It might indeed seem sufficient to say merely that those parts of stem which receive the cambial stimulus survive, instead of saying that they are protected from being killed. But, in the Leguminous seedlings at least, the killing of stems that do not receive the cambial stimulus is as a general rule somehow actively brought about by the growing leaves in another part of the plant. For the writer decapitated and defoliated the main shoots of three Pea seedlings and continually removed all buds from them, and found that the stems were

* The contrary opinion was put forward by Neef (1914, pp. 526-529) for strips of bark of Lime shoots. But he does not state that his upward-pointing strips regenerated anything more than a comparatively small amount of wound-wood and callus, so that it is not clear on what grounds he based his opinion.

still alive and healthy after periods of 81, 53 and 53 days, when the experiment was stopped. In these plants no growing leaves were present anywhere, and the results may be contrasted with those of the previous experiments (Snow, 1931, b), in which, in Pea plants with two equal shoots, if one of them was defoliated, it was regularly killed by the other shoot for the greater part of its length after 28 days or less, at approximately equal temperatures. The way in which inhibited shoots are killed will be briefly discussed in Section 7.

4. *The Elongation of Young Downward-pointing Strips.*

The next step was to investigate the growth of downward-pointing strips similar to those of the last experiment, but cut out from young elongating internodes instead of fully-grown internodes. This was done as follows:—

Experiment 3.—Eight seedlings of *Vicia Faba* were grown until their third or fourth internode was about 4 or 5 mm. long. Then this internode was split as accurately as possible in the median plane of the leaves with a very fine scalpel, the leaf below it having been removed, to make the operation possible. The split extended through the whole internode and just through the node below it. One of the half stems so formed was cut through transversely, as before, at the level of the base of the split, so that it formed a downward-pointing strip attached at the top only. The following table shows the length

Table II.

Plant number.	Length of split internode at start, in millimetres.	After 6 days.		After 14–15 days, when fully-grown.	
		“Cut” half.	“In line” half.	“Cut” half.	“In line” half.
1	4.5	10.5	13.75	16.5	20.5
2	5.75	9.5	10.0	13.25	15.5
3	5.0	12.0	9.0	18.0	13.25
4	5.0	9.25	9.5	10.25	12.5
5	5.0	9.0	9.0	15.0	14.25
6	4.5	9.0	9.0	17.0	14.25
7	4.0	8.25	6.5	11.5	9.5
8	5.0	10.75	11.0	15.0	15.0
Means.....	4.88	9.78	9.73	18.56	18.33

in millimetres of the half internode included in this strip and also of the other half internode, at the start and after various times. The former is called the “cut” half, and the latter the “in line” half. The measurements were made

from the node at the base of the split internode to the top of the split. After a week or more, the "cut" halves began to curve geotropically, but they were then loosely tied to the other halves to prevent this, vaseline having been put on the cut surfaces.

The table shows that the downward pointing "cut" halves grew at practically the same rates as the "in line" halves, and reached on the average practically the same final lengths. It therefore seemed of interest to repeat the experiment on still younger internodes as follows.

Experiment 4.—In 10 seedlings of *Vicia Faba*, young internodes, only from 2.0 to 2.75 mm. long, were operated upon in the same manner as the internodes in the last experiment. The following table shows the results, arranged as before.

Table III.

Plant number.	Length of split internode at start, in millimetres.	After 12-16 days.		After 20-24 days, when fully-grown.	
		"Cut" half.	"In line" half.	"Cut" half.	"In line" half.
1	2.5	8.25	10.5	10.0	12.5
2	2.0	11.25	12.75	26.5	26.5
3	2.5	8.75	15.0	19.5	22.5
4	2.0	5.75	7.5	6.25	7.5
5	2.25	7.25	9.0	9.25	11.0
6	2.75	8.0	10.25	11.25	16.5
7	2.25	18.5	16.5	25.0	26.0
8	2.25	6.5	11.0	10.25	15.0
9	2.5	5.5	11.0	15.25	19.5
10	2.75	10.5	15.0	14.5	16.25
Means.....	2.37	9.02	11.85	14.77	17.32

This table shows that even when the split internodes were only from 2.0 to 2.75 mm. long at the start, the downward-pointing "cut" halves grew only a little less rapidly than the "in line" halves. Their mean rate of growth during the first 12 to 16 days was, on the average, 70 per cent. of that of the "in line" halves, and their total growth was 83 per cent. of that of the latter. They also grew rather less than the "in line" halves in nine of the 10 plants. The rates of growth varied greatly in the different plants, which were not very uniform in vigour.

The above two experiments show that the downward-moving stream coming from the apical region of the shoot includes all that is needed for the rapid elongation of young internodes from an early stage onwards. But they do not by themselves show that the downward-moving stream is able to protect

elongating internodes from being inhibited. For the elongating strips were close beneath the growing leaves, and it was shown previously (1931, *a*, p. 221) that close beneath these leaves there is in any case little or no inhibition; for at distances less than about 10 mm. beneath them even axillary buds are scarcely inhibited, if at all. Nevertheless, comparison with the results of the previous paper (SNOW, 1931, *b*, p. 313) makes it sufficiently clear that some growth-promoting influence coming from the growing leaves does protect elongating internodes from being inhibited. It was there pointed out that in plants with two equal shoots, the growing leaves of each shoot protect their own shoot from being inhibited and killed by the other shoot; for they protect the mature parts of their own shoot from being killed, and also its elongating internodes from being inhibited in growth by the other shoot. Since it is the cambial stimulus moving downwards from the growing leaves which protects mature parts from being killed, as was shown above, it is natural to conclude also that it is either the cambial stimulus, or some other growth-promoting influence coming from the growing leaves, which protects the elongating internodes below them from being inhibited. This conclusion makes it necessary to withdraw the alternative explanation of these results suggested previously (*loc. cit.*) in which it was supposed that the inhibiting influences travelling down the two shoots were somehow of a polar nature, and so counteracted one another when they met: for it is now seen that the explanation offered above is better supported by the evidence.

It is now possible to answer the question how it is that the growing leaves of an intact shoot promote the growth of their own shoot below them, but tend to inhibit and kill another defoliated shoot. For firstly as regards the mature zones, they transmit the cambial stimulus to their own shoot below them and thereby protect it from the inhibiting influence which they also transmit. But the cambial stimulus cannot travel upwards into the defoliated shoot, whereas the inhibiting influence does travel upwards into it, and consequently that shoot is killed. Secondly as regards the elongating zones, the growing leaves do not inhibit those of their own shoot since they are close beneath them, and at short distances even the axillary buds are scarcely inhibited. But they do inhibit the elongating zones of another defoliated shoot, since those are far enough away. Reasons will, however, be given in the next section for considering that the absence of inhibition from the regions close beneath the growing leaves may be only apparent, and due in turn to another growth-promoting influence, which, travelling only for a short distance from the growing leaves, co-operates in protecting those regions from being inhibited.

5. *Internodal Elongation, and the Increase of Inhibition with distance.*

The results of experiments 3 and 4 make it difficult to accept an explanation of the "increase of inhibition with distance," which was mentioned previously as one possible explanation (Snow, 1931, *a*, p. 222). For it was there suggested that the growing leaves might inhibit the axillary buds by modifying conditions in the shoot in such a way that nutritive or growth-promoting substances tended to flow upwards away from the lower regions and concentrate in the uppermost region, with the result that in this region the buds would not be inhibited and the young internodes would be accelerated in elongation, as actually happens. But the above experiments show that strips of internode elongate rapidly even if cut off below from any upward flowing stream at a very early stage. Consequently the *downward*-flowing stream must be enough for their rapid elongation, and the explanation mentioned would at least need modification.

However, the following facts suggest incidentally another way of explaining the puzzling increase of inhibition with distance. There is in the shoot another growth-promoting influence besides the cambial stimulus, for Tammes showed (1903) that in many woody species each leaf strongly increases the elongation of the neighbouring two or three internodes, and further that its influence often extends upwards as well as downwards. In *Vicia Faba* and *Pisum sativum* also the writer finds that each developing leaf accelerates the elongation of the next internode below, though less strongly than in the plants studied by Tammes. Whether each leaf also accelerates the elongation of the next internode above is difficult to determine in these plants, but in *Pisum* it seems to do so slightly. Now reasons were given in the last section for considering that *some* growth-promoting influence coming from the growing leaves protects the young internodes from being inhibited, and it seems very probable that it is this elongation-promoting influence which is partly or mainly responsible for protecting them. But since in the species studied by Tammes the elongation-promoting influence, unlike the cambial stimulus, can travel upwards and since there is no evidence that it travels very far, it may be expected that in the Leguminous seedlings, in which it seems to move in the same manner, it will to some extent travel up into the axillary buds that are fairly close to the apex of the shoot, and also that originating from the growing leaves, it will fail to reach the lower parts of the shoot. As a result the buds near the apex will be more or less protected from inhibition, but those lower down will not be protected. However, it is not possible to explain in this way the apparent

increase of inhibition with distance that was found in the lower parts of the shoot in Child's experiments, mentioned previously (Snow, 1931, *a*, p. 219).

It is of interest that in many shoots at least, the elongation-promoting influence travels upwards as well as downwards, whereas in coleoptiles the growth-promoting substance that can be extracted from the tip can scarcely travel upwards at all (Went, 1928, p. 58). Yet it has been shown that in *Helianthus* and *Asparagus* the elongation-promoting influence, since it can pass a watery gap (Beyer, 1925 ; Oosterhuis, 1931), is also probably due to a hormone formed near the tip of the shoot.

6. *The Position between Growing Leaves and Roots.*

It remains to be determined to what extent the fate of a portion of stem depends on the second of the three factors mentioned in the introduction—namely, whether it is in, or out of, the line between growing leaves and roots. It has indeed been shown already that these differences of position are of practically no importance for the parts that receive the cambial stimulus ; for they survive and grow in thickness about equally fast in either position. But it has still to be determined whether the fate of parts of stem that do *not* receive the cambial stimulus depends on whether they are in the line between growing leaves and roots, or, as is more usual, out of it. The following experiment was carried out partly for this purpose.

Experiment 5.—Ten seedlings of *Vicia Faba*, forming series A, were grown in a mixture of sand and sawdust until their main roots were about to produce secondaries or had just begun to do so. Then the main roots were deprived of their tips and split in half in a plane at right angles to the plane of insertion of the cotyledons, and the seedlings were replanted in pots. The half roots formed vigorous secondaries, and healed their cut surfaces by regeneration. Later, when the seedlings had expanded their third or fourth leaf, they were decapitated in the internode above this leaf, and deprived of all axillary buds except the bud in the axil of one cotyledon, which was left to grow out (see fig. 3A, which represents one of these plants a few weeks later, when this bud had grown into a shoot). A few days after the decapitation, when the remaining bud had grown only a few millimetres, the split was continued upwards from the root through the cotyledonary node and the lower part of the main shoot, as shown in the figure, so that the halves of the decapitated main shoot now formed a line of tissue connecting the growing axillary with one half of the root system and one cotyledon. Yet they were not in a position to receive

from the axillary the cambial stimulus, since this could not travel upwards into them.

As controls (fig. 3B), 10 similar seedlings, forming series B, were operated upon in the same way, and then the halves of their decapitated main stems that were the farther from the remaining axillary buds were cut through near the base, just above the cotyledon. Consequently the halves of the main

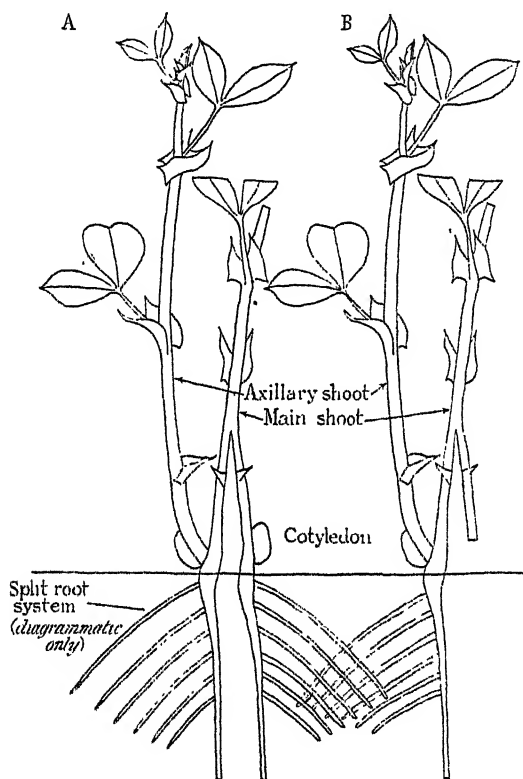


FIG. 3A AND 3B.

stems of these plants were not in the line between growing leaves and roots, and their farther halves were left as downward-pointing strips attached at the top. The plants of the two series were nearly always operated on in pairs at about the same date, and were closely matched in vigour. The halves of the split stems, above the cotyledons, were from 33 to 50 mm. long and the parts above the splits were considerably longer. The cut surfaces above ground were vaselined.

The method described above was adopted for the last six pairs of plants. In the first four pairs the roots were not split until the same time as the stems,

at which time the splitting is more awkward. The plants were all grown in summer, in a frame beneath which the air was kept fairly moist, and mineral fertiliser was supplied when the cotyledons became exhausted. The conditions were favourable, as was shown by the fact that nearly all the growing axillary shoots retained all, or nearly all, their leaves in healthy condition, even after they had expanded twelve or more. The leaves of the decapitated shoots were reduced in area, to diminish transpiration, and after a few weeks they were still further reduced, or removed, if these shoots began to wilt on hot days, as many of them did. This slight wilting was quite distinct from the processes leading to death, since the shoots did not become discoloured, and later recovered completely.

The results were briefly as follows. Amongst the plants of series A, in all except one the stem parts of the decapitated shoots were still alive and healthy when the experiment was stopped. In two of them, they had then survived for 78 and 76 days after the completion of the operation, in one for 63 days and in six for times from 51 to 56 days. (The numbers of leaves that had expanded meanwhile on the axillary shoots ranged from 11 to 16.) Only in one plant did the decapitated shoot die: in this one its farther half became discoloured just above the cotyledon after 45 days, and a few days later most of the rest of it was discoloured and dying. In this plant the farther half of the root also began to die at about the same time, whereas in the other plants both halves of the root system were alive and healthy at the close of the experiment, except in one, in which the farther half root was beginning to die at 56 days. On the other hand, in the 10 plants of series B, the most distant parts of the decapitated shoots—that is, the farther split halves and the most apical parts above the split—began to turn yellow and die after times ranging from 31 to 45 days, when the numbers of leaves expanded on the axillary shoots were only from 6 to 11. Soon afterwards, usually in less than a week, the discoloration spread to the nearer split halves also.

The halves of the decapitated shoots, therefore, survived much longer in the “A” plants than the “B” plants. The total amount of root system was not very different in the plants of the two series, for the halves of the root systems beneath the axillary shoots grew and branched strongly, whereas the farther half root systems of the “A” plants grew very little after the main shoot was decapitated. But in the “A” plants, the halves of the main stem formed a line of tissue connecting the growing leaves of the axillary shoot with part of the root system, and, for several weeks, with one cotyledon: these farther cotyledons became exhausted after from 21 to 35 days in eight of the “A” plants,

in the other two they lasted to the end. It therefore seems probable that the position of the half stems of the "A" plants protected them by saving them from being drained of some substances necessary for life. But a point that makes the interpretation difficult is that the upper parts of the decapitated shoots also survived in the "A" plants, and it is doubtful to what extent these can be said to have been in the line between growing leaves and roots. The nature of the process by which inhibited shoots are killed will be further discussed in Section 7. In any case, the position between growing leaves and roots is a much less important factor than the cambial stimulus, since at most it only enabled the stems to survive in rather a precarious condition, and not to grow in thickness and form new conducting tissue, as is pointed out below.

In the "B" plants, the farther halves of the split main stems and the apical parts above the split were the parts that consistently began to lose colour and die the soonest. After the farther halves had begun to lose their colour, the times that elapsed before the nearer halves also began to lose colour, ranged from 2 to 10 days, the mean being 5 days. These facts support the conclusion reached in Section 2 that the susceptibility of a portion of stem to the inhibiting influence coming from the growing leaves of another shoot, does not depend on its orientation. For though the killing of a stem is a different process from the inhibition of its growth in length, it nevertheless follows upon the process of inhibition. Consequently if the orientation of the downward-pointing farther halves had protected them from the inhibiting influence of the axillary shoots, they would not have begun to die until after the nearer halves, whereas actually they always began to die sooner.

The half stems of four of the "A" plants were examined microscopically, and it was found that there had been a little regeneration beneath the cut surfaces: for small patches of tissue had been formed by divisions at the cut edges of the ring of cambium, and the cells of the pith had enlarged at right angles to the surface and divided tangentially a few times. The regeneration was slightly stronger in the farther than in the nearer halves. It was only a little more than in one of the upward-pointing strips of experiment 2, and there was no sign of a new cambium spreading across the pith, nor, so far as could be judged, did the old cambium appear to have grown appreciably. The roots also were examined in three of those "A" plants in which they had been split at the same time as the stems, and it was found that in the farther half roots there had been practically no cambial growth or regeneration, whereas the nearer halves, which received the cambial stimulus from the growing axillary shoots, had grown very strongly and regenerated complete rings of cambium.

It is therefore clear that in those parts that do not receive the downward-moving cambial stimulus the cambia do not grow appreciably, even if they are in the line between growing leaves and roots.

The same point is shown still more clearly by the following experiment (experiment 6) illustrated in fig. 4. The main shoots of six seedlings of *Vicia*

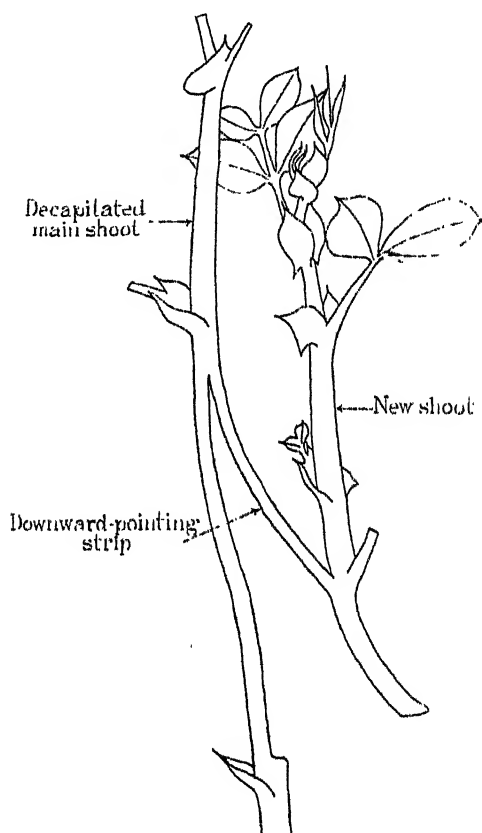


FIG. 4.

Faba were decapitated and split in the manner shown in the figure, and a bud was left at the node on the downward-pointing strip, all other buds having been removed. The leaf at this node was also removed. The part of the strip below the bud was from 15 to 30 mm. long, and the part of it above the bud about 30 mm. The bud then grew out and formed a shoot, as shown in the figure. It expanded two leaves, or sometimes three, but then the halves of the main stem between it and the roots collapsed from lack of water (without dying or becoming discoloured), although the air was kept moist. The plants were then all examined (after about 30 days, in March), and it was found that

throughout the parts of the strips of stem below the new shoots the old cambia had grown very strongly, and there had also been strong regeneration at the cut surfaces, new cambia having begun to spread across through the new tissue formed from the pith. But in the parts of the strips above the new shoots and in the other half stems there had been practically no cambial growth or regeneration, only a very few divisions at the cut edges of the cambia. Yet these parts were the only tissues connecting the new shoots with the roots and cotyledons, and until the new shoots expanded their leaves practically all the materials for their growth must have travelled to them through these parts. The regenerated tissues below the new shoots were sharply delimited from the parts above them.

7. The Killing of Inhibited Shoots.

A few points need to be noted concerning the causes of death in inhibited shoots, though the present investigation was not directly concerned with this question. The death of these shoots is preceded by a large loss of dry weight. The writer has found that in Pea seedlings stumps consisting of two internodes of decapitated main stem, beneath which an axillary bud is growing out, if picked and dried when their leaves have scarcely begun to discolour (after 15 to 19 days), weigh much less than similar stumps of decapitated stems, after the same times, beneath which no bud is growing out. On the average, the dry weight per centimetre of five of the former stumps was only 58 per cent. of that of five of the latter. Further, the results of experiment 5 make it quite probable that the loss of dry weight not only precedes the death of the inhibited shoots but is actually the cause of it.

The writer therefore cannot agree with Mogk (1913), who maintained that the death of inhibited shoots cannot be due to starvation. Mogk's chief reason for this statement was that in inhibited shoots the most apical parts die first, whereas in starved shoots the basal parts die first. But the writer deprived seven Pea seedlings of their cotyledons, and placed them in very dim light to die of starvation, and found that in five of the seven the upper parts of the stems died first, just as in inhibited stems. However they did not turn yellow, as inhibited stems do, but died and shrivelled while still green. Moreover there is a curious point of resemblance between inhibited and starving shoots. For in growing pea shoots each young leaf, between the limits of about 1 mm. and 20 mm., is roughly three times longer than the next younger. But in inhibited shoots one often finds a sudden jump from a leaf of about 2 mm. to a leaf of 10 or 12 mm. or more—as if the larger leaves have continued growing

for longer than the smaller ones. Now in four of the seven starving shoots referred to above, just the same phenomenon was noticed. But even if the death of inhibited shoots is due to withdrawal of substances, it does not follow that the earlier process of inhibition of growth is due to the same cause. Moreover, the withdrawal of substances, if it is the cause of death, must depend on the previous intervention of physiological factors, since the most distant parts regularly die first. On the other hand, when detached parts of plants are emptied of nutritive reserves by simple diffusion from their basal cut surfaces, the basal parts are emptied first, as would be expected (Puriewitsch, 1898).

8. Conclusions.

The main conclusions have been fully stated in sections 3, 4 and 5, but will here be briefly summarised and discussed. The question was raised how it is that the young leaves near the apex of a shoot tend to inhibit lateral buds or shoots, but promote the growth of their own shoot below them. The answer indicated is that the young leaves transmit downwards various growth-promoting influences which override their inhibiting influence in the parts which they reach, but do not penetrate into lateral shoots or buds (except possibly those that are close to the apex), whereas the inhibiting influence does penetrate into them. Of these growth-promoting influences, the best known is the stimulus for cambial growth, which does not penetrate into lateral buds or shoots since it cannot travel upwards; this stimulus protects the mature parts of the shoot from being killed. The growing leaves also protect the young internodes of their own shoot from being inhibited in their elongation, and it is probable that they do so partly or mainly by means of the promoting influence which they are known to exert on internodal elongation. This influence seems as a general rule to travel only for a short distance, and consequently it need not be expected that it will reach lateral buds or shoots that are more than a short distance away. But since, unlike the cambial stimulus, it is able, in many plants at least, to travel upwards it may enter the lateral buds that are very close beneath the growing leaves. If so, this will help to explain the otherwise puzzling fact that actually these buds (within 5 or 10 mm. from the apex in *Pisum*) are scarcely inhibited at all.

These provisional conclusions resemble to some extent a suggestion made by Loeb (1924, p. 101 and p. 108) concerning the inhibiting effect exerted by apical leaves on axillary buds below them in stem cuttings of *Bryophyllum*.* For he

* Ossenbeck (1927, p. 342) did not detect this effect. Many of his results with *Bryophyllum* were different from those of Loeb.

suggested that the leaves promoted the growth of the stem below them in length or thickness, and that the growth of the stem in turn inhibited the axillary buds, either by withdrawing nutriment or in some other way.* If this "growth of the stem" is interpreted in the sense of cambial growth, the suggestion is a valuable one, which deserves more attention than it has received. Certainly it becomes a little surprising, if Loeb's suggestion is accepted, that in the Leguminous seedlings inhibition can travel upwards with little or no loss of intensity, in lateral shoots into which the cambial stimulus does not penetrate (Snow, 1931, *a*, p. 217). But, apart from this point, it is possible to explain the present results just as well on the basis of Loeb's suggestion as in the way proposed above. However, even if Loeb's suggestion should prove correct, it would still be necessary to conclude that cambial growth or other processes of growth promoted by the growing leaves in the shoot below them somehow protect the shoot from inhibition. For otherwise one would not be able to explain how it is that two equal shoots continue growing rapidly if intact, though if either is deprived of its growing leaves it is rapidly inhibited by the other.

Throughout the present paper indeed, the writer has for simplicity spoken of the inhibiting influence as starting from the growing leaves. But previously, when it was shown that it is the growing leaves which inhibit the axillaries, it was carefully pointed out that they may do so indirectly, by means of the cambial growth which they stimulate in the stem (Snow, 1929, p. 348). If this should prove to be the way in which they inhibit, then the conclusions of this paper, as stated above, would not disagree with Loeb's suggestion. The point at issue is simply whether the inhibiting influence (in a narrower sense) originates as such from the growing leaves or from the cambium below them which they stimulate into growth. If it should turn out to be the growing cambium which inhibits, then it would be necessary to consider whether it does so by withdrawing substances necessary for growth. For though strong objections have been brought against the older and very different theory that the inhibition of axillaries is directly due to the consumption of materials by the shoot apex, many of these objections would not hold against the suggestion that materials are withdrawn from axillaries to the cambium of the main stem near their insertions. However, any such withdrawal from an axillary shoot would need the previous intervention of physiological factors, as was pointed out in Section 7. Also, if it were the growing cambium which inhibited, then the

* When commenting previously (1925) on Loeb's experiments, the writer had not noticed this suggestion.

writer's earlier results (1925), which showed that one stage of the whole inhibiting process can travel down through the pith and pass a protoplasmic discontinuity, could be interpreted as applying to the first stage in the process, the downward transmission of the cambial stimulus.

The conclusions of this paper may also be compared with those of Umrath (1931), who has recently been led to assume an antagonism between a hormone promoting elongation and an excitatory hormone, in order to explain the interesting fact that repeated stimulation of *Mimosa* leaves causes the shoots to elongate less and branch more.

Summary.

(1) The question is raised how it is that, in Dicotyledons, the growing leaves of a shoot tend in general to inhibit lateral buds or shoots, but promote the growth of their own shoot below them.

(2) The difference is not directly due to the fact that the lateral buds or shoots are differently orientated with regard to the influence reaching them from the growing leaves of the dominant shoot.

(3) In *Vicia Faba* strips of mature stem that are out of the line between growing leaves and roots survive and grow in thickness if they are attached by the top, so that they receive the downward-moving cambial stimulus; but if attached by the base, they die. Also strips of very young internodes, attached by the top only, elongate rapidly.

(4) The fact that the stems of growing shoots are in the line between growing leaves and roots is only of subordinate importance: for parts of stem that are in this line do not make any cambial growth unless they receive the cambial stimulus, though they survive much longer than similar parts that are not in this line.

(5) The conclusion drawn from these and other facts is summarised and discussed in Section 8. It is briefly that the stems of growing shoots are protected from the inhibiting influence by the cambial stimulus and other growth-promoting influences coming from their own growing leaves.

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A Closer Analysis of the Heat Production of Nerve.

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The heat production of nerve is believed to occur in two phases, "initial" and "recovery"; the former is presumably an accompaniment of the physical and chemical changes which take place during the propagation of the impulse; the latter, of the processes by which those changes are reversed and the nerve restored to its initial state.

It is not easy to separate the one from the other; indeed, during the earlier part of this research it was realised that in a strict sense, and on the evidence available, there might really be no "initial" heat at all. This is made clear by the following discussion.

Let us suppose that the rate of heat production in the recovery process following a single impulse obeys the relation given in curve A, fig. 1, falling rapidly from the start and then more slowly, reaching zero some considerable time (several minutes) afterwards. All the analyses given below agree in two characteristics, viz. (i) a rapid decline in the rate of recovery heat production during the first few seconds after the end of stimulation, followed by (ii) a very prolonged and slowly falling phase. These common characteristics are most easily explained by supposing that recovery occurs in two stages, one of high initial rate but falling rapidly, the other of low initial rate but falling slowly.* Let us assume that each factor is represented by an exponential curve, so that the rate of recovery heat production at time t after an impulse is

$$y = Ae^{-at} + Be^{bt}. \quad (I)$$

In the following numerical description of the nerve impulse at 20° C. we will take $A = 160$, $B = 16$, $a = 1.6$, $b = 0.004$. A and a refer to the initially-rapid and rapidly diminishing process; B and b to the slow and slowly diminishing one.

* There are two such separate processes in recovery after muscular contraction, (i) delayed lactic acid formation providing energy for phosphagen reformation, this is rapid; and (ii) oxidative restoration, which is slow.

It is not suggested that this equation fits the recovery process of nerve precisely. It does, however, provide the two factors required, and is, in

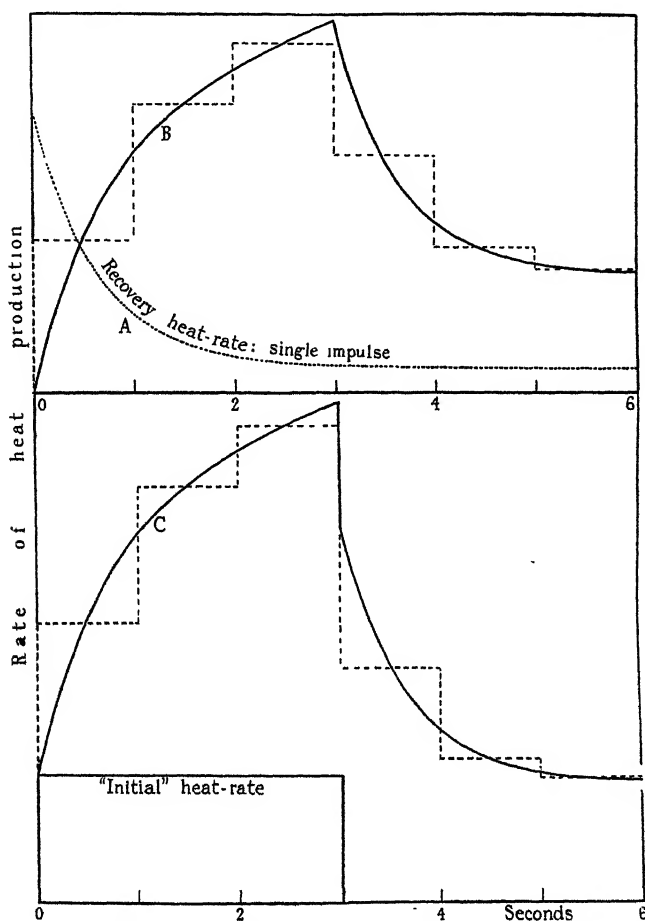


FIG. 1.—Calculated rate of heat production of nerve in 3-second "stimulus." *Upper*—Without including initial heat. *Lower*—With initial heat at a constant rate as shown. Equations given in the text. The calculation assumes that the rate of recovery heat production after a single impulse obeys the relation shown in curve A (*upper*) which has the equation $y = Ae^{-at} + Be^{-bt}$. The blocks of heat (broken lines) are drawn to show how the rate of heat production would appear from an analysis, each block being equal in area to the curve for the interval of time on which it stands. Note that the first block does not represent the initial heat, which is much less in the lower figure and nothing at all in the upper figure. Note also that the sudden step down at the end of the stimulus is not equal to the sudden step up at the beginning of the stimulus. [It would be equal were the step down measured from the top of a block during a 4th second of stimulation to the top of the 4th block when stimulation ends at 3 seconds.]

general, a correct description of them. Its great advantage is that it allows a simple mathematical deduction to be made of the form of the curve relating rate of heat production to time, in and after a stimulus of any duration.

Let us suppose that in a stimulus of duration T seconds successive impulses sum together in respect of the heat they produce, and that during constant stimulation they are of constant size. This again may not be strictly true, but the error (if any) is not so great as to affect the general argument. We may deduce an equation for the rate Y of recovery heat production, during and after a stimulus of given length T , by integrating* the above equation as follows :—

$$\begin{aligned} Y &= \int_0^t \{Ae^{-a(t-\theta)} + Be^{-b(t-\theta)}\} d\theta \\ &= \frac{A}{a} (1 - e^{-at}) + \frac{B}{b} (1 - e^{-bt}). \end{aligned} \quad (\text{II})$$

This equation applies during the stimulus. After the stimulus, similarly, since integration is now only to time T ,

$$Y = \frac{A}{a} (e^{-a(t-T)} - e^{-at}) + \frac{B}{b} (e^{-b(t-T)} - e^{-bt}). \quad (\text{III})$$

In fig. 1 (*upper*) is a curve (B) drawn from equations (II) and (III) with the constants given above, to represent the case of 3 seconds stimulation. This curve is very similar to the results of the analysis of short stimuli, shown below in figs. 2, etc.

So far we have introduced no initial heat at all. It is instructive, however, to split the heat up into 1-second blocks (as shown by the broken lines), each block being equal to the total heat during the second on which it stands. If the three blocks during stimulation had been obtained by actual analysis we should probably have called the first of them the "initial" heat. The sharp drop at the end of stimulation might similarly have been attributed to the sudden end of the initial heat. It is noticeable that the step down at the end is appreciably less than the step up at the beginning; it might have been concluded that the initial heat diminishes during the stimulus. And yet we have assumed a case with no initial heat at all.

Let us now introduce initial heat at a constant rate (50 units per second) during the stimulus, see fig. 1 (*lower*). The blocks, drawn as before, show a

* Strictly speaking, for any given frequency n of stimulation we ought every second to take the *sum* of n elementary curves distributed at intervals of $1/n$ second. In all practical cases, however, n is so large that the *sum* can be replaced by an integral.

greater step up at the beginning, and a greater step down at the end. It would still be impossible, however, without an analysis in much shorter time units than 1 second, to decide how much of the first block is initial heat, how much recovery.

Fig. 2 shows an actual analysis of the heat produced in a 16-seconds stimulus at 19.2° C. Details are given in the legend. It is clearly impossible to decide

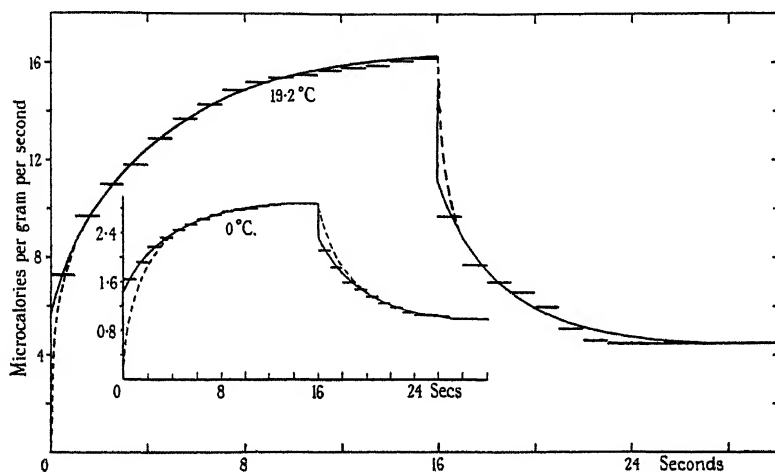


FIG. 2.—Nerve heat production at 19.2° C. January 25, 1932. Analysis, by 1-second heating control, of the mean of 27 records, not all maximal, of the response to 16 seconds stimulus in oxygen. Various frequencies. Results multiplied up in the ratio (mean height of greatest 4 records) : (mean height of all 27 records) = 1.33, to represent a maximal response. Records taken at about 5-minutes intervals on the same day as the nerves were dissected. Zc galvanometer without amplification. For record see fig. 7, A, for analysis Appendix I, C. The results of the analysis are shown by short horizontal lines. The full line drawn through them assumes a sudden rise in heat production when stimulus begins and a sudden fall when it ends. The broken line starting from the origin and joining the full line further up, and the broken line falling continuously from the moment the stimulus ends, assume that the heat production rises from zero continuously—not suddenly—at the beginning of stimulation, and falls continuously—not suddenly—when it ends. It is impossible to decide with certainty whether the full line, or the broken line, fits the results more accurately. *Inset*—Experiment of February 9, 1932, at 0° C. on cold acclimatized frogs. 16-seconds stimuli, various frequencies, mean of 24 records analysed by 1-second heating control and multiplied up to correspond to maximal stimulus. Nerves used on same day as dissection. Note that times are plotted on half the scale and heat on $2\frac{1}{2}$ times the scale of the high temperature experiment. The full line and the broken line are drawn as in the main figure. In this case the full line fits the results far more accurately than the broken line; indeed, it is impossible to draw the curve to the origin without assuming large errors in the analysis. For analysis see Appendix I (D).

(as it was with the blocks of fig. 1) whether the rate of heat production rises suddenly, or gradually, from the origin, whether it falls suddenly, or gradually, when stimulation ends. Inset in fig. 2 is a smaller curve, in this case of a 16-seconds stimulus at 0° C. At this temperature, compared with processes occurring in the nerves, the instruments are relatively much quicker, the 1-second intervals of analysis much shorter. Consequently, a finer analysis is possible and it is evident that there is initial heat after all. Without the experiments at 0° C., however, the reality of the initial heat would have remained in question.

The initial heat as a separate entity was first described by Downing, Gerard and Hill (1926), who employed as a measure of it the maximum deflection of a galvanometer connected to a thermopile on which a nerve, stimulated for 10 seconds, lay. This deflection was reached in about 17 seconds, and a large part of what they called "initial" heat must certainly (it now appears) have been recovery heat following the earlier impulses set up. They found the "initial" heat to be about 11 per cent. of the whole heat; it might be less: it could not be more.

Gerard (1927), employing more rapid recording instruments, undertook an analysis of the heat in time units of 1 second; his results indicated a gradually rising rate of recovery heat production superimposed on a constant rate of initial heat production during and ending with the stimulus. The analysis in 1-second units of the data obtainable at that time must have been very precarious, and in any case, as we have just seen, such an analysis at room temperature leaves the reality of the initial heat in doubt. Gerard again estimated the initial heat as 11 per cent. of the whole.

Bronk (1931), employing a still more rapid thermopile and galvanometer, analysed the heat in time units of 3 seconds, and found the same result, viz., recovery heat continuing for many minutes superimposed on initial heat produced at a constant rate during the stimulus. His figures were more decisive, the rapid rise and fall at the beginning and end of stimulation being quite clear; even so, however, the heat during each 3 seconds—the apparent initial heat—might be made up largely—as we shall see that it was—of recovery heat starting very soon after the earlier shocks of the 3 seconds in question.

Bronk's quicker apparatus allowed him to reduce the initial heat from 11 per cent. to 9 per cent. of the whole. Would much more rapid recording reduce the initial heat to a still smaller fraction, or perhaps to nothing at all?

The question is of fundamental importance in discussing the nature of the nervous impulse. If this be merely a self-propagating electrochemical change,

the momentary electromotive effect at an "active" point somehow inducing similar momentary "activity" at a neighbouring point, and this at the next, etc., the initial heat might be no more than that associated with a slight redistribution of electrolytes inside and outside the fibre. The energy in the observed electric change is small: even if the assumptions underlying its calculation (Hill, 1921) be not entirely justified, it is scarcely possible by any others to make it much larger*; and the redistribution of electrolytes, caused by a momentary change of permeability, could have but a negligible thermal effect. On the other hand, the reinstatement of ions in their initial position might require a prolonged "secretory" activity, and lead in consequence to a relatively large heat production. The efficiency in secreting, of the kidney at any rate, is certainly low (*e.g.*, 1 to 2 per cent.: see Borsook and Winegarden, 1931). To take a simple model, mixing M/10 KCl with M/10 NaCl gives very little heat; to separate them, however, by any actual process, involves a considerable expenditure of energy. Were the initial heat really found to be a negligible fraction of the whole the electrochemical theory of the nervous impulse would receive strong support.

In crustacean nerve (Hill, 1929) the initial heat is in fact a much smaller fraction of the whole than was estimated in frog's medullated nerve, *viz.*, about 2 per cent. Closer analysis, by means of more rapid recording instruments, might easily reduce the true initial heat still more. It was curious, in any case, that crab's limb nerve and frog's medullated nerve should differ so largely. It was desirable, therefore, to find out whether, and how far, the "initial" heat would survive investigation by finer and more rapid methods of recording.

The task was a formidable one, and had to wait for improvements in thermopile and galvanometer. If the heat was to be analysed in shorter intervals the apparatus had to be made more sensitive, since in shorter intervals less heat is produced, and at the same time it had to be made quicker, more capable of following a rapid change. In the design of instruments in general, sensitivity and quickness are antitheses, sensitivity varying inversely as the square of speed. Random disturbances, moreover, whether intrinsic or extrinsic, have a greater influence on rapid than on slow recording systems. Ultimately, as it proved, for the shorter intervals of stimulation, one had to push the sensitivity and speed of the galvanometer so far that "Brownian" oscillations of its moving system caused appreciable unsteadiness in the records; beyond this it

* See, however, *note added in proof*, p. 156 below.

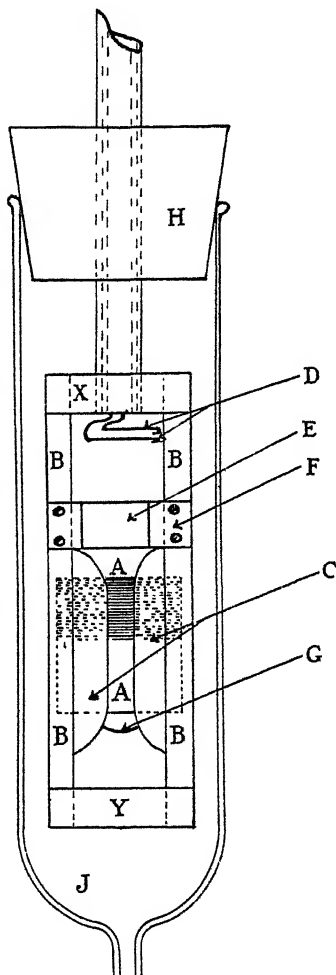
was useless to go, increase of speed or sensitivity, by reason of greater unsteadiness, would give no real advantage. Such "Brownian" oscillations might be abolished by placing the whole recording system (including the thermopile) at a very low temperature, *e.g.*, in liquid air; apart from any other objection to such drastic treatment, the presence of living nerves on the thermopile prohibited it.

There being therefore an absolute limit to the galvanometer sensitivity available for a given speed, it was necessary to increase the sensitivity of the thermopile. A new instrument was designed in consultation with Mr. A. C. Downing and constructed by him, and the success of the present investigation must be attributed largely to his skilful work; see fig. 3. This thermopile has a far lower resistance and a rather greater thermo-e.m.f., while it retains its heat much better than previous patterns, and since nerves are placed on both sides of the thermo-junctions (not on one side only as in earlier designs) it is quicker in its action. Another instrument has been constructed, but not in time for the experiments described here; it gives 50 per cent. more c.m.f. for the same heat, and has a far smaller correction for "heat leak" from the stimulating electrodes.

The fundamental difficulty in answering the criticism embodied in fig. 1, viz., that the supposed "initial" heat may really be early recovery heat, is that the instruments are not rapid enough to follow the course of the heat production during the first half second of a tetanus. In muscle advantage has been taken of the fact that cooling the tissue slows all its reactions, thus greatly increasing the relative speed of the recording instruments. In muscle, where one's instruments are sensitive enough to deal with a single twitch, no increase of sensitivity is required at the lower temperature—rather the reverse, since the single response is greater than at a higher one. In nerve, however, where a succession of impulses is required (the immediate rise of temperature for a single impulse at 20° C. being about 7×10^{-8} °C.) it was foreseen, and it actually proved to be the case, that less heat is produced, for a given duration of stimulus, at a low temperature than at a high. Whereas, at 20° C. the nerve increases its heat response up to a frequency of 300 to 400 per second, at 0° C. a maximum is reached already at about 30 per second; assuming a maximal impulse to give the same heat at both temperatures, the total initial heat per second would be only about 1/10 at the low temperature. This expected diminution of the heat by a low temperature prevented one from going immediately to 0° C. as a means of increasing the relative speed of the instruments; actually when it became clear that the problem was insoluble at a high tempera-

ture the conditions at the low were found to be rather less exacting than expected, owing to the fact that a single impulse at 0°C . gives much more heat than at 20°C .; and a decision has been reached, and doubts as to the existence of the initial heat removed, by experiments at the low temperature.

FIG. 3.—Nerve thermopile, two-thirds actual size. A, sheet of 150 couples of constantan-iron (hard-soldered), the "hot" junctions being down the middle line, the "cold" junctions alternately on either side and insulated by a thin sheet of mica and by bakelite from the brass frame B. The groove containing the hot junctions is formed between two walls C of paraffin wax. There are similar paraffin wax walls and a similar groove on the opposite side. D, stimulating electrodes drawn up as far as possible. E, large silver plate built up at the back with silver blocks, held by the bakelite clips F. The silver plate E cuts off heat conducted from D towards A, and acts also as the upper warming electrodes during calibration. G, lower warming electrode, consisting of a thick silver wire. H, rubber stopper forced into glass tube J, which has a hole at the bottom to admit gas, or to withdraw Ringer's solution, as required. Another tube leading through the stopper for the admission of gas is not shown. The whole of the brass frame is thickly insulated with bakelite varnish; the thermopile is completely insulated from the brass frame, as also are the silver plates and electrodes; and the whole is waterproofed by dipping in hot paraffin wax to make a thin film on its surface. This film is very thin on the thermopile face but is renewed by melting with a minute gas flame before each experiment. The nerves are held in place during an experiment with threads stuck by plasticine to X and Y.



The Galvanometer.

During the earlier experiments and in many of the later ones a Zernicke Zc moving coil galvanometer, constructed by Messrs. Kipp en Zonen of Delft, was employed without amplification. This admirable instrument, which is similar to—but rather more sensitive than—that used in the previous study of crab's nerve (Hill, 1929), was mounted on a Julius suspension, and could be read directly to 0.1 mm. on a scale at 2.2 m. (or if desired at $3\frac{1}{2}$ m.) distance.

On windy days it showed slight disturbances, and such days were avoided as far as possible for the experiments. The Julius suspension, which was damped by a vane in oil below, was adjusted so that the centre of gravity of the whole hanging system was approximately at the point from which the moving coil was suspended by its quartz fibre; it was so effective that even violent impulses to the wall from which the system hung caused no disturbance at all. The damping should be only just sufficient, otherwise vibrations are communicated through the oil.

The galvanometer is of about 25 ohms resistance and its magnetic shunt was adjusted to give just critical damping with 50 ohms external resistance (that of the thermopile used). At 2.2 m. distance with the thermopile in the circuit 10^{-6} volt gave about 40 mm. deflection, so that $1 \text{ mm.} = 10^{-6}/40 \times 75 = 3.3 \times 10^{-10}$ amp. about, which was readable by estimation to 0.1 mm. on the scale, *i.e.*, to 3.3×10^{-11} amp. The period of the instrument (undamped), when oscillating on open circuit, was 5.4 seconds.

It is now recognised that galvanometers must ultimately show "molecular" or "electronic" movements if made sufficiently sensitive, and it is useless to push their sensitivity beyond the limit at which these begin to produce measurable disturbance. This was discussed, particularly in relation to moving-coil galvanometers, by Ising (1926) and the disturbances visible after sufficient magnification by the thermo relay (Moll and Burger, 1925) were shown to be due to unavoidable molecular causes.

Sensitive moving magnet galvanometers are similarly limited (Hill, 1926). If $\bar{\theta}$ be the root mean square deflection (in radians) of the moving system from its zero position, MK^2 its moment of inertia, t_0 its complete period, then

$$\bar{\theta} = \frac{3.2 \times 10^{-8} t_0}{\sqrt{MK^2}},$$

For an actual galvanometer constructed by Mr. Downing, MK^2 was 2.25×10^{-6} , so that $\bar{\theta} = 2.1 \times 10^{-5} t_0$. Taking t_0 as 4, a period sufficiently short for the present experiments, $\bar{\theta} = 8.4 \times 10^{-5}$, so that the mean displacement of the reflected beam would be 1.68×10^{-4} radian = 0.37 mm. at 2.2 m. distance. Since it is easily possible to read photographic records from such a galvanometer at such a distance to 0.1 mm. (see Hartree, 1931) it is clear that the limit set by molecular movements has already been reached. In his work on the heat production of single twitches Hartree uses a similar galvanometer at $2\frac{1}{2}$ m. with a period of 0.75 second. Here $\bar{\theta} = 1.6 \times 10^{-5}$ and the calculated mean disturbance on the scale is 0.08 mm. Since Hartree reads his records

to 0.1 mm. he is clearly working near the limit of his galvanometer's performance.

According to Ising (1931), if \bar{i} is the mean deflection of a moving coil galvanometer from its average position, calculated in amperes, R the total resistance of the galvanometer circuit in ohms (the galvanometer being assumed critically damped), T_0 the undamped period in seconds, and n the fraction of the damping due to electromagnetic causes,

$$\bar{i} = \frac{1.2 \times 10^{-10}}{\sqrt{nRT_0}} \text{ amp.}$$

Considering the case of the Zc galvanometer described above, for which $R = 75$, $T_0 = 5.4$, $n = 0.823$, we can calculate, $\bar{i} = 6.1 \times 10^{-12}$ amp. The galvanometer was readable directly on the scale by estimation to 0.1 mm., i.e., to 3.3×10^{-11} amp. Its photographic records, taken at $4\frac{1}{2}$ m., could be read to 1.6×10^{-11} amp. Clearly it had not reached its limit, so far as molecular movements were concerned, and amplification, if available, could be usefully employed.

Electron tube amplification for such small e.m.fs. as are here available has never been satisfactorily developed. In the present investigation the heat liberated as the result of 0.5 second of stimulation at 20° C. was measured, and the e.m.f. produced was about 2×10^{-8} volt. It would require peculiarly stable valves and batteries to allow reasonably smooth records to be taken when 0.02 microvolt was all one had to measure. Amplification by the thermo-relay introduces too much lag to be permissible, in a case where the greatest quickness is required; a linear thermopile used in the same way as the relay (see Gerard, 1927) has less lag, but still too much. Just before the present experiments began, I was fortunate enough to be shown by Dr. P. Auger, of the Institut de Biologie physico-chimique in Paris, a cuprous oxide photoelectric cell which it was possible to employ, in place of a thermo-relay, for amplifying one galvanometer by means of another. Dr. Auger was kind enough to supply me with several cells for the purpose, and all the experiments at high sensitivity have been made with a pair of these.

The method is easily applied (see Hill, 1931). The beam of light from the primary galvanometer connected to the thermopile falls symmetrically on two opposed halves of the photoelectric cell, or on two similar cells placed side by side. These are connected to the secondary galvanometer. When the beam of light from the primary galvanometer moves, the cell produces a current in the secondary, the magnitude of which is proportional to the movement. The

cell itself has no measurable lag and its current is so large that a relatively insensitive (and therefore rapid) secondary galvanometer can be used, the amplified deflection being almost identical with the primary one. A 30-fold amplification is readily obtained, beyond which—owing to random disturbances—it is useless to go.

In the earlier experiments, before photographic recording was adopted, two methods were employed: either (1) the deflection of the moving coil galvanometer was read directly to 0.1 mm. on a scale, or (2) it was amplified by the photoelectric cell used with a Downing moving magnet galvanometer of about 50 ohms resistance. It was found impossible to read and record the deflection at intervals less than 3 seconds, and this is not good enough for an accurate analysis of the early stages of the heat production. Signals were given every 3 or 6 seconds by a Jacquet clock connected to an amplifier and a loud speaker, and the observer read and recorded the deflection at each signal. For the longer stimuli this was sufficient.

For more accurate analysis, however, photographic recording was necessary, and for this the Downing galvanometer, used as a "secondary," was replaced by another Zernicke moving coil of shorter period than the primary. The beam of light came through a narrow slit, fell on the mirror, and was reflected into a "plate camera" of the type used with the string galvanometer. In the dark slides bromide paper was used, covered with sheets of glass; this is cheaper and more convenient than photographic plates. Time signals were made on the records every $\frac{1}{2}$ or 1 second by intercepting the light with a metronome.

For the records of the shorter stimuli (see figs. 11 and 12), where quickness of response was the first consideration, the Zc galvanometer was replaced by a Zd of 3.6 seconds period;* with this it could be calculated that the mean disturbance due to molecular causes was equal to the deflection produced by 0.7×10^{-11} amp. The amplified system was used at a sensitivity of 1 mm. = 2×10^{-11} amp., so that the mean molecular disturbance should be about 0.3 mm.: actually on a quiet day photographic records showed a mean deviation from a smooth line of about 0.5 mm (see fig. 8A). This unsteadiness, and others, were discounted by taking a large number of records and analysing their mean.

* [Added in proof.—It has been found possible to amplify a Zb galvanometer of 1.8 sec. period, employing a Moll galvanometer of 1.3 sec. period as the secondary. This has not yet been used for the present purpose; its use should appreciably sharpen the analysis.]

The Preparation.

Ten sciatic nerves dissected out as long as possible from five large Hungarian frogs (*R. esc.*) were used in each experiment. Threads were attached to their ends. They were mounted on the thermopile alternately on either side, the threads to their lower ends being held to the bottom of the thermopile frame by "plasticine." From the point where they left the thermopile face at its lower edge the nerves were bent round the lower "warming" electrode and usually a thread was tied below the latter round all of them to keep them firmly in position. No considerable uninjured portion of the nerves lay beyond the warming electrodes, and the thread tied tightly round all the nerves together below the electrodes further ensured that at the lower end the heat by control warming was limited to about the same region as the heat by activity. The rather thick silver warming electrode, moreover, must be fairly effective in thermally shielding the nerve below it from the nerve above it. The control heating, therefore, from the lower electrode upwards, is probably sufficiently comparable with the "live" heat production.

At the upper end of the thermopile the 10 nerves, 5 from each side, came together and after crossing a gap of about 4 mm. passed on to the face of the heavy silver block provided to cut off the heat from the stimulating electrodes. Thence they crossed a further gap of 13 mm. to the stimulating electrodes. They passed above one of these and below the other, and the threads to their upper ends were then fixed with plasticine to the top of the thermopile frame. The nerves were drawn just tight so that they remained firmly in position without strain. A thread was tied very gently round them between thermopile and silver block, to keep them the better in place.

The silver block was used as the upper warming electrode; the control heat, therefore, was liberated from the lower edge of this downwards. The "live" heat production, on the other hand, extended right up to the stimulating electrodes and it might seem that a different spatial distribution of "live" and "control" heat would cause appreciable error in the absolute values, or in the analysis. The silver, however, carries away very nearly all the heat set free in the nerves lying on it; it would not otherwise be so effective as a thermal shield. Hence, even were heat set free during the controls in the part lying on the silver it would not reach the thermopile or affect the result; the controls, therefore, are adequate. In calculating the absolute values, ergs or micro-calories per gram, the weight of nerve considered is that lying between the warming electrodes.

After the nerves had been mounted a glass cover was placed over the thermopile and filled with Ringer's fluid buffered with phosphate to p_H 7.2 (2 mg. P/100 c.c.). A thick rubber tube led from the bottom of the glass cover and another from the pipe at the top of the instrument. By these oxygen could pass through the solution, or the latter could be removed and the chamber filled with oxygen or nitrogen as required. The nerves were always in oxygen, or nitrogen, during the actual observations, and the chamber was completely closed.

Thermostat.

The complete arrangement was then sunk in a large Dewar flask filled with water or paraffin oil. The flask had been mounted in a box filled with cork dust; this box was then placed in a second, larger box filled with cork dust, a funnel-shaped space being left above the flask and boarded in at the sides. The second box had a felt-lined cover dropping into the top of the funnel and a close-fitting lid. It in turn was placed in a still larger box also filled with cork dust and supplied with another close-fitting lid. Leads for the thermopile, for the stimulating electrodes, and for the warming electrodes, were permanently fixed in the funnel-shaped space, as also were rubber and glass connections to the outside for the tubes to the nerve chamber. The conical space above the Dewar flask was closely packed with cotton wool, as also was that between the covers of the second and third boxes.

The water, or the oil, in the Dewar flask was stirred by a slow stream of air or gas.

In early experiments an attempt was made to use a thermostat employing a gas-regulator maintaining the bath temperature constant to within 0.001°C ., as read by a thermometer graduated in 0.002°C . (see Hill, 1930, p. 12). It was found, however, that with the nerve thermopile employed the disturbances were so violent that this thermostat had to be abandoned. The thermopile is not symmetrical, as was the previous type (Downing and Hill, 1929) to some degree, and so cannot be expected to be unaffected by temperature fluctuations. In spite of the relatively good stirring, and the nearly continuous adjustment afforded by grinding the gas jet on the slant, fluctuations of temperature do occur, too rapid or too small to be detected by the thermometer, and these caused the violent disturbances observed.

Much better results indeed were obtained with a simple Dewar flask gently stirred and standing in the room. Its temperature might not be constant but

at any rate it changed continuously. The superiority of the simple Dewar flask over the "constant" temperature bath led, therefore, to the adoption of the arrangement described above, in which the Dewar flask was very carefully lagged. This was the most successful method discovered of avoiding temperature disturbances, particularly when the temperature of the room was maintained approximately constant by means of a pentane regulator, a pair of electric relays and an electric radiator.

In the experiments at 0° C. the Dewar flask was filled with crushed ice and stirred with a slow stream of air.

Curious disturbances were sometimes witnessed when the thermostat was slowly cooling, or after a gas, which had bubbled through a wash-bottle in the room, was introduced into the chamber at 0° C. Without warning, but usually after the stimulating circuit had been touched, a large prolonged deflection occurred in the heating direction, which one could only attribute to a "rain-storm" in the chamber, started off probably by an electric charge on the stimulating wires. At more ordinary sensitiveness such disturbances might be disregarded; at that of the present experiments they could make observations impossible. The condensation of 1 mg. of water *per day* would give out five times the "initial" heat (at 20° C.) of 180 mg. of nerve stimulated continuously; the "total heat" in a 1-second stimulus, say 1.2×10^{-4} cal. per gram, or 2.4×10^{-5} cal., could be equalled by the deposition of 4×10^{-8} g. of water. It was necessary to avoid such disturbances. At high temperatures, therefore, the room temperature was arranged to be slightly above that of the thermostat, so that in the chamber the temperature tended rather to rise than to fall, and super-saturation did not occur. At 0° C. dry oxygen was introduced directly from a cylinder, avoiding the wash-bottle.

Another curious disturbance is caused by compression of the gas in the chamber. With a chamber volume of 150 c.c. a pinch of the rubber pressure tubing connecting it to the outside might, at high sensitivity, cause a deflection of several hundred millimetres. This is quite intelligible when we reflect that an adiabatic change of volume of one part in a million should cause a change of temperature of about 0.0001° C.—enough, at the highest sensitivity used to send the spot of light about 500 mm. Factors of which ordinarily one takes no account may be serious when one is working at sensitivities between 0.5 km. and 3 km. per 1° C. Puffs of wind beating on the building, the slamming of doors, a movement of the rubber tube connected to the chamber, may easily cause pressure changes large enough to spoil a record. In all experiments, therefore, the chamber was completely sealed up, the thick rubber tubes being

closed with taps, and so far as possible windy and disturbed days were avoided for the experiments.*

Stimulation.

In all previous work on nerve heat production stimulation has been by means of an induction coil. There are three disadvantages in this :—

- (a) Stimuli may not be quite constant in strength, or in frequency, and they cannot be exactly reproduced.
- (b) The energy in the stimulus is not exactly known, as is required if an accurate allowance is to be made for the leak of heat from the stimulating electrodes.
- (c) The character of the stimulus, as defined by the rate of rise and fall of the current, is not known and so it is not possible to use a stimulus of minimum energy.

Of these the last mentioned is very important since, particularly in the case of longer stimuli, heat leak from the stimulating electrodes cannot be altogether avoided and therefore the energy of the stimulus should be made as small as possible. For all three reasons stimulation by coil can with advantage be replaced by stimulation by condenser discharges, employing an arrangement more or less as described in a recent paper (Hill, 1931, *a*) ; see also Bozler (1931). Using a commutator capable of going at comparatively high speed it is possible to vary the number of shocks per second administered to the nerve from a maximum of over 500 (250 charges, 250 discharges) down to as low a value as required. Using charges as well as discharges, polarisation at the electrodes is avoided and successive stimuli start alternately at either electrode. The capacity of the condenser employed, and the potential to which it is charged being known, the energy in the stimulus is exactly defined and stimuli are exactly reproducible. Moreover, by varying the capacity, or the resistance in series with it, the rate of discharge can be altered at will so that a stimulus of minimum energy can be employed.

As long ago as 1899 Waller showed how to determine the stimulus of minimum energy, employing condenser discharges. The energy of a condenser discharge is $5 F V^2$ ergs, where F is the capacity in microfarads, V the potential in volts. The minimum of this, for an effective stimulus, occurs at a certain rate of

* *Added in proof.*—Another thermostat is being constructed in which the thermopile is to be surrounded by three metal cylinders of high thermal conductivity, separated by non-conducting media, and to be shut off entirely from the atmosphere.

discharge and the time occupied in the discharge is proportional to RF , where R is the resistance in series with the condenser. From Waller's results on frogs' nerves (presumably English *R. temp.*) a value of RF for minimum energy may be calculated, namely, about 580.

Lucas (1906, *b*) investigated the "optimal stimulus" to the sciatic nerve of the toad, and from his results the "optimal" value of RF , at 10°C. , may be calculated, viz., 1500. Lucas (1906, *a*) similarly examined the sartorius of the frog, and for what he later called the γ substance of this (presumably nerve) the "optimal" value of RF , at 10°C. , may be calculated, viz., about 330. The frog is a quicker animal than the toad, so the value is less.

A few experiments were performed on sciatic nerves of Hungarian frogs (*R. esc.*), the animals used throughout the present research. Employing electrodes of silver 6 mm. apart, minimal stimuli just exciting the nerve and causing a small response in the muscle were determined. The resistance of the nerve between the electrodes was about 10,000 ohms and this was short-circuited by 1000 ohms, so that the total resistance in series with the condenser was 900 ohms. For a given voltage the least capacity required for excitation was found, and FV^2 calculated. The voltage was changed and the process repeated. The least value of FV^2 was determined in this way, and so the value of RF for minimum energy. In various experiments at room temperature ($17\frac{1}{2}^{\circ}\text{C.}$) the following values of RF were obtained: 285, 183, 266, 133, 320, mean (say) about 250.

The curve relating the energy required to excite to the capacity is very blunt in the neighbourhood of its minimum, so that any value of RF reasonably near the minimum should be good enough.

It is necessary, in employing this criterion of minimum energy in the stimulus, to know the resistance in series with the condenser. The resistance of the nerves is not exactly known, nor is it convenient to determine it when the nerves are alive and the experiment is ready to begin. It is better to short-circuit the stimulating electrodes through a resistance sufficiently small to render the nerve conductivity negligible, and to make this the more certain it is best to place in series with the nerve a fairly high resistance. In all experiments at room temperature 5000 ohms was placed in series with the nerve lying on the stimulating electrodes, and the total resistance, presumably about 7000 ohms, was short-circuited by a resistance of 1010 ohms. Thus R , the total resistance in series with the condenser, was about 875 ohms. At 0°C. , where the excitation time of the nerve is greater, RF must be greater too; a rather larger capacity therefore was employed, while a resistance of

10,000 ohms was placed in series with the nerve and the whole short-circuited through 2000 ohms.

There is another way of varying the energy required for stimulation, namely by varying the distance between the stimulating electrodes. According to Rushton (1927) the potential gradient v required for excitation is given by the equation

$$v = \frac{1}{1 - e^{-s}},$$

where s is the distance between electrodes. The equation applies to a nerve bathed in salt solution. The difference of potential therefore is vs and the energy is $v^2s^2/(\text{resistance})$, which is equal to v^2s^2/ks , where k is some constant. This is proportional to

$$\frac{s}{(1 - e^{-s})^2}.$$

Differentiating to find the minimum value, we obtain

$$e^{-s} = \frac{1}{1 + 2s},$$

which is true when $s = 1.25$ approximately. Rushton measures his distances in "analytical units" which at room temperature are about 5 mm. Thus s , the distance between electrodes for minimum energy, is about $6\frac{1}{4}$ mm.

This calculation was submitted to Dr. Rushton, who criticised it on two grounds, namely (i) that resistance is not directly proportional to length as assumed, and (ii) that whereas his results were obtained for stimulation in Ringer's fluid they have been applied here to the case of stimulation in air. He was kind enough to undertake a theoretical treatment of the case in air and concluded that it would be safe to halve the result obtained above and to use an interpolar distance of about 3 mm. The actual distance employed with the thermopile shown in fig. 3 was $3\frac{1}{2}$ mm. It is unlikely that, so far as variation of inter-electrode distance is concerned, the conditions were far from those in which the energy of the stimulus was a minimum.

In spite of all these precautions to work with stimuli of as little energy as possible, the heat produced by the stimulus may still be a formidable factor unless special methods are adopted to deal with it. The electrodes therefore were withdrawn as far as possible from the thermopile, very long nerves being employed, obtained from the largest available specimens of Hungarian *R. esc.* Even so, however, the heat liberated by the stimulus at the electrodes is conducted down to the thermopile unless steps be taken to cut it off. A large

block of silver, therefore, was placed about 4 mm. above the thermopile on the electrode side, into which so much of the heat as was conducted that far might be carried. It was found, however, at first, when this silver was connected directly to the frame of the thermopile, that the heat so carried away was conducted into the frame and reached the "cold" junctions of the thermopile, causing large negative deflections. The silver, therefore, was removed from direct metallic connection with the frame and was held in place on pieces of bakelite, which is a poor conductor of heat, in the hope thereby of cutting it off from the frame. This was a great improvement and was all that could be done with the apparatus used in the present research. Even so, however, with longer stimuli, *e.g.*, of 32 seconds and more, heat is conducted to the thermopile from the stimulating electrodes and allowance must be made for it as described later. In a new apparatus recently constructed by Mr. Downing, with a larger block of silver, the "heat-leak correction" is much less.

With stimuli of short duration the effect of heat leaking from the stimulating electrodes is of negligible importance—in fact in most cases it could not be detected at all. With such delicate electrical measurements, however, there is always a danger of electrical disturbances, particularly those from the stimulating circuit, causing errors too large to be neglected. For this reason in a number of experiments the nerves were asphyxiated at the end, and when asphyxia was complete the stimulus, as used in the observations on the live nerve, was applied again to test its effect. In every case the galvanometer remained completely unaffected by the application of the "stimulus" to the asphyxiated nerve. Only if the stimulus be continued for a longer time is heat observed to leak from the stimulating electrodes.

This freedom from electrical disturbances was achieved by various devices :—

- (A) The electric motor driving the commutator lay on an earthed zinc sheet so as to avoid leaks from the mains.
- (B) The whole of the stimulating arrangement—motor, commutator, condensers and battery—was placed some distance away in the next room.
- (C) An earth line was connected to the galvanometer circuit.
- (D) The observer stood on an insulated board and avoided touching anything during an observation except the insulated handle of a Morse key.
- (E) No current from the electric mains was used in the room during an experiment except for the ordinary lighting. Motors, etc., were disconnected and the Pointolite lamp was run on accumulators.

- (F) No electrical arrangements, other than a simple short-circuit key worked by the observer, were used to determine the duration of the stimulus. A metronome giving the light signals for the photographic record was set going and the duration of the stimulus was determined by the observer opening a short-circuit key at one signal and closing it again at the required subsequent signal. The metronome ticked seconds or half-seconds and it was found by trial, of a key connected to an electro-magnet writing on a smoked drum, that the intervals so given by hand, eye and ear were sufficiently accurate.
- (G) Other people were not allowed to move in, or near, the room during a record.
- (H) The room was kept warm and dry to avoid films of moisture on the apparatus.

With all these precautions it was found that photographic records, even at extreme sensitivity, showed no detectable disturbance when the stimulus used in the actual observations was applied to nerves rendered inexcitable by deprival of oxygen. In this freedom from disturbances the method of stimulating by condenser discharges is far superior to that involving the use of a coil, where the spark of the contact breaker produces high alternating potentials which tend to get rectified and can with difficulty be induced not to cause disturbance.

In 1927 Gerard, Hill and Zotterman studied the effect of frequency of excitation on the heat production of nerve. They had considerable difficulty, mainly because of disturbances, and the curve they gave was not from a single experiment but the mean of a large number. With the present arrangement the frequency of stimulation can be varied over a wide range with ease, and the freedom from disturbances is such that a good curve relating heat to frequency can be obtained from a single experiment; see fig. 5.

Stimulation with Minimum Energy.—Preliminary trials on gastrocnemius-sciatic preparations had shown that the criterion for stimulation with minimum energy was that at room temperature (17.6°C.) the product RF (resistance in ohms \times capacity in microfarads) should be about 250. Two possible factors might disturb the application of this result to the present experiments: (*a*) in the muscle-nerve experiments only motor nerve fibres were considered, while in the nerve heat experiments all the fibres, sensory as well as motor, took part, and (*b*) single stimuli on the one hand, and long series of stimuli at high frequency on the other, might yield different results. It was desirable

therefore to make experiments directly on nerve heat, finding, for constant energy in the stimulus, the value of RF for which the greatest response was obtained.

Two such experiments are shown in fig. 4, one at 21.3°C ., one at 0°C . The value of F (the capacity in microfarads) was varied, V (the voltage) being adjusted so that the energy of the stimulus, which is proportional to FV^2 , was constant; at each value of F , the heat produced by the nerves, in a stimulus

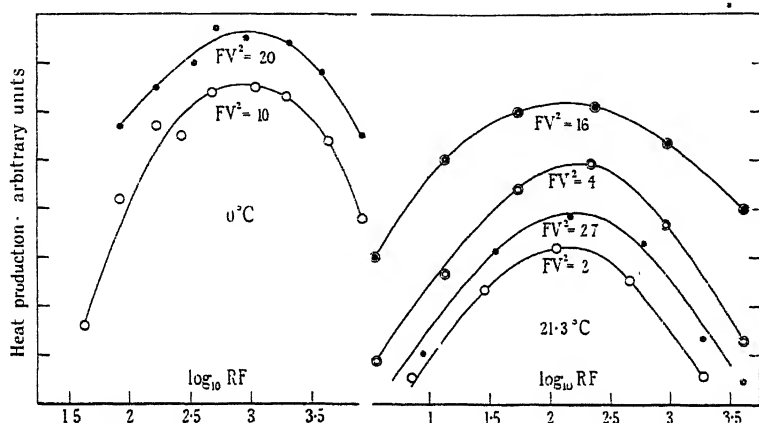


FIG. 4.—To find the stimulus of minimum energy. Two experiments at 0°C . and 21.3°C . respectively. Vertically, heat production of nerves (arbitrary units), in 16 seconds (0°C .) and 12 seconds (21.3°C .) stimuli. Horizontally $\log_{10} RF$, where F (mfd.) is the capacity of the condenser used for stimulation and R (ohms) is the total resistance in series with it; RF is proportional to the charge or discharge time of the condenser, *i.e.*, to the duration of the single shock. In each series the energy of the stimulus (which is proportional to FV^2) was kept constant, F and V being changed, and response to a stimulus of given energy varied as shown with the character of the shocks employed. The greatest response to a stimulus of given energy was given (*a*) at 0°C . when $RF = 900$, (*b*) at 21.3°C . when $RF = 160$, *i.e.*, with times to half discharge of 0.58σ and 0.11σ respectively. In these experiments the values of R were 1720 ohms and 885 ohms, at 0°C . and 21.3°C . respectively, and the frequencies 22.6 and 310 shocks per second.

of given duration, was measured from the maximum deflection. Photographic records were made. The heat was plotted as a function of $\log_{10} RF$, R being constant throughout an experiment.

The result is clear. The heat response, for a given energy in the stimulus, varies with the capacity, and has a maximum at a capacity which is the same for different values of the energy. In the nerves at 0°C . (from cold acclimatized frogs) the value of $\log RF$ at maximum response, for given energy in the stimulus, is about 2.95, RF therefore about 900; in the nerves at 21.3°C .

(from warm acclimatised frogs) the maximum is at $\log RF = 2.2$, RF therefore about 160. In the cold experiments R was always taken about 1720 ohms, so the optimum capacity was about 0.5 mfd.; in the warm experiments R was always taken about 885 ohms, so the optimum capacity was about 0.18 mfd.

The maxima in fig. 4 seem to be rather sharp; this, however, is due to the manner of drawing the curves. If heat be plotted against capacity, not against its logarithm, a very blunt maximum is obtained; a rather large variation of the capacity, on either side of the optimum, *e.g.*, doubling or halving it, has a comparatively small effect. It is sufficient that the capacity employed should be in the region of the optimum. If, however, the product RF be very far from the optimum the curves show how inefficient the stimulus becomes. It is clearly important in nerve heat measurement, where heat leak from the stimulus may always be appreciable, to ensure that an optimal stimulus is used. This is easy employing condensers, difficult or impossible by any other method. With crabs' nerves, which require a strong stimulus in any case, the use of an optimal stimulus in the way described above will be particularly beneficial.

The value $RF = 160$ (for 21.3°C.) corresponds to a condenser discharge half completed in 0.11σ ; the value $RF = 900$ (for 0°C.) to a discharge half completed in 0.58σ . These apply to nerves from large Hungarian frogs (*R. esc.*) with metal electrodes $3\frac{1}{2}$ mm. apart; with other nerves and with other electrodes the optimum rate of discharge may well be different, and should be determined anew.

The symmetry of the curves in fig. 4 is curious. Why is the heat response, which is presumably proportional to the number of fibres responding, so nearly a symmetrical function of the logarithm of the time occupied by a stimulus of given energy? There may be no significance in the answer, but the question is worth asking. An analogous symmetry is seen in Lucas' curves (1906).

Frequency of Stimulation.—The effect of frequency on the heat production of nerve was studied by Gerard, Hill and Zotterman (1927); it was of interest to confirm their results with the present equipment, employing condenser discharges as stimuli.

It is necessary that the shocks should be complete, or nearly complete, in the intervals between them. In the experiment at 21.3°C. shown in fig. 5, the capacity used was 0.158 mfd., which was discharged through 885 ohms; hence $RF = 140$. In 0.001 second (*i.e.*, at 1000 per second) the charge or discharge $(1 - e^{-10^6/RF})$ is complete to within 0.08 per cent., which is more

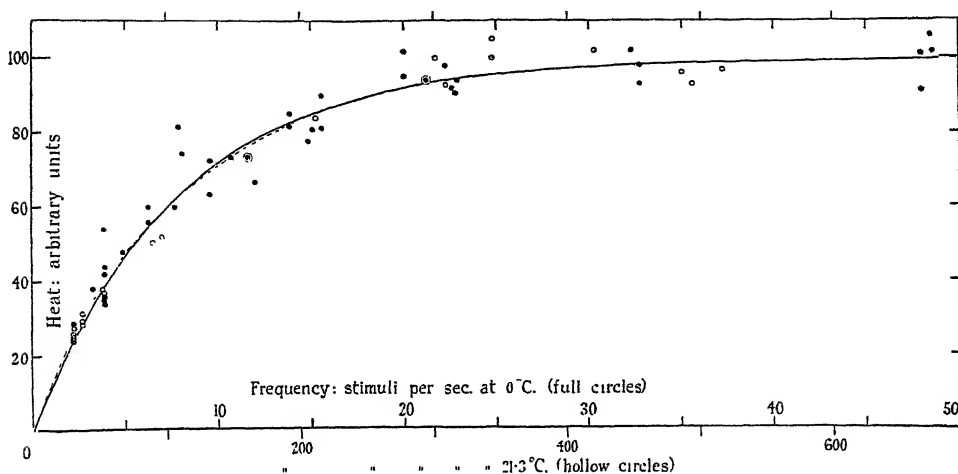


FIG. 5.—Relation between heat production of nerves and frequency of stimulation. Two exactly comparable experiments at 0° C. (February 9, 1932, full circles) and 21.3° C. (February 3, 1932, hollow circles), each on 10 nerves from cold acclimatized and warm acclimatized frogs respectively. Vertically, heat produced by nerves, arbitrary units chosen to give a mean value of 100 at high frequencies. Horizontally, condenser charges and discharges per second; scales chosen to make the mean curves at the two temperatures coincide. Stimuli: at 0° C., 16 seconds, 10.2 volts, 0.616 mfd., $R = 1720$ ohms; at 21.3° C., 12 seconds, 12.5 volts, 0.158 mfd., $R = 885$ ohms. Zernicke (Zc) galvanometer, no amplification; photographic records; heats given by maximum deflections from extrapolated baselines. The full line drawn through the observations is an exponential curve, $y = 100(1 - e^{-kx})$, where at 0° C. $k = 0.127$, at 21.3° C., $k = 0.00915$. The broken line drawn arbitrarily fits the observed points slightly more closely.

than sufficient. It is necessary also that stimuli should be strong, lest in the relatively refractory phase at high frequency some of the shocks should fail.

Charges as well as discharges were employed; this avoided polarisation and possible leaks to the recording system, and it gave double the frequency of stimulation for a given speed of the commutator. The nerves were in good condition at both electrodes (which were $3\frac{1}{2}$ mm. apart), and presumably impulses started equally from both with the rapid condenser discharges employed.

The heat was measured in the earlier experiments directly on the scale, with amplification, in the later experiments by photographic recording, without. The maximum deflection was taken as a measure of the heat. It is realised that only under certain conditions is this justifiable. When, however, the duration of stimulus is the same throughout an experiment, when successive readings are taken in regular sequence (so that the nerves are in a steady state),

when in consequence the deflection curves are of the same shape though of different size, and when absolute values are not required but only a comparison of one reading with another, then the maximum deflection is the best (and far the easiest) measure of the magnitude of the heat.

It is not necessary to describe the many experiments in detail. The last pair, however, at 21.3° C. and 0° C. respectively, deserve some attention. They were made with special precautions, the former on frogs which had been kept in the room, the latter on frogs kept in the cold store, for some days beforehand, so as to become "acclimatized" to the conditions of the experiment. The nerves were dissected and set on the thermopile early in the day, and the experiments made in the afternoon as soon as thermal equalisation had occurred and disturbances had disappeared. The two experiments are strictly comparable, and since 10 nerves were used in each a fair average was obtained.

Further details are given in the legend of fig. 5. The results of the cold experiment, which are the more numerous, are shown by full circles, those of the warm experiment by hollow circles. The latter, however, are confirmed by those of several other experiments not shown here.

The scales of heat were chosen to make both sets of observations converge to the same maximum of 100 per cent. at high frequency; the scales of frequency to make the two sets of points lie around the same mean curve. It was not a foregone conclusion that the latter adjustment was possible; it might well have been the case that the relation was quite different at different temperatures, so that no adjustment of scale would make the results coincide. Fig. 5, however, shows that such a difference does not exist. As usual, indeed, a change of temperature has simply altered the scale of time on which the reactions of the tissue occur. (See *e.g.* Feng (1931, fig. 1) for the case of muscle.)

The effect of temperature is rather large: 1 second in the nerves at 0° C. corresponds to 0.072 second in those at 21.3° C. If we venture to calculate an ordinary temperature coefficient we obtain $Q_{10} = 3.45$. From the point of view, however, of physical chemistry the calculation is doubtful, because the cold experiment was made on cold acclimatized frogs, the warm on warm acclimatized frogs. It was not possible to perform the experiment at 0° C. on "warm" frogs since their nerves reacted so poorly in the cold that the heat readings were too small to measure accurately. It is clear, however, that there is a large effect of temperature on the time-scale of the heat-frequency relation, and that apart from this the results at different temperatures are essentially the same.

The full curve drawn through the points of fig. 5 is a simple exponential one

$$\text{Heat} = 100 (1 - e^{-k (\text{frequency})}).$$

For nerves at 0°C ., k was 0.127 ; at 21.3°C ., 0.00915 . This purely empirical relation is convenient in describing the form of the curve; for example, three other experiments at 20°C ., 17°C . and 19°C . gave values of k :—

$$0.0090, \quad 0.0094, \quad 0.0086; \quad \text{mean } 0.0090,$$

agreeing closely with the value 0.00915 . No importance, however, can be attached to the comparative accuracy with which the equation fits the observed points. Indeed, for accuracy of fit, the broken line (drawn arbitrarily) is rather better.

The physical basis of the relation shown in fig. 5 was discussed by Gerard, Hill and Zotterman (1927). It depends upon the rate at which a nerve "recovers" (in the anaerobic, not the aerobic sense) after an impulse has gone by. In fig. 6, calculated from the broken line of fig. 5, the heat per impulse is plotted as a function of the interval between impulses. The curve,

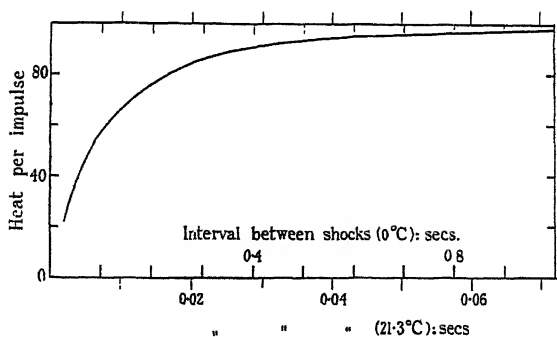


FIG. 6.—To show the recovery in capacity to produce heat in response to a shock, as a function of the interval between shocks. Calculated from the broken line of fig. 5. Vertically, heat per impulse; horizontally, interval between successive shocks (2 scales, for 0°C . and 21.3°C . respectively). The physical basis of the curve is discussed in the text and by Gerard, Hill and Zotterman (1927).

exponential in general type, is of a complex nature, since the nerve is made up of individual fibres, each recovering at its own rate. No doubt it would be possible, by assuming a probability distribution of the fibres, supposing each to recover along an exponential curve, and allowing each a certain period of breakdown and absolute refractoriness before recovery begins, to calculate

a relation similar to that of fig. 6. No useful purpose, however, would be served by so doing. The existence of the relation found between the energy set free and the interval allowed for recovery is a sufficient indication at present of what is happening in the nerve; further refinements are not possible on the evidence available.

The Analysis.

Controls.—In the earlier experiments the analysis of photographic records was at first carried out with an “instantaneous” heating control, records being made of the heat ($5FV^2$) set free when a condenser of given capacity (F mfd.), charged to a given potential (V volts), was discharged through the nerves. These records afford a means of calibrating in absolute units (see Bozler, 1931; Hill, 1931, *a*, p. 279) as well as of analysis.

The “instantaneous” control rises very rapidly, and is well suited to the analysis of records, as of muscle heat (see Hartree, 1931), in which the rate of heat production starts at a very high value and then decreases rapidly. It soon appeared, however, that in nerve the conditions are so different that a different method is required. The “initial” heat production occurs during the stimulus at a constant, or a gradually decreasing rate, and superimposed on it is the gradually increasing recovery heat production. The changes of rate, except instantly at the start and end, are not very rapid, and they can best be analysed with a control made by heating the nerves for a time equal to the interval of analysis. This type of control was used in the two latest studies of nerve heat (Hill, 1929, *a*; Bronk, 1931) and was finally adopted throughout in this.

The interval of analysis was always the same as the duration of the control heating. In a few early experiments where “instantaneous” controls had been made the following procedure was adopted: a $\frac{1}{2}$ second control was made by adding an instantaneous control starting at time 0 second to another starting at $\frac{1}{2}$ second; a 1 second control by adding instantaneous controls starting at 0 second, $\frac{1}{2}$ second and 1 second. Usually 1 second controls were adopted, occasionally for special purposes $\frac{1}{2}$ second, 2 seconds or longer. For a typical 1 second heating control record see fig. 7D. It is always possible to build up the longer controls from the shorter by addition (see Hill, 1929, p. 169).

In making a control record for (say) 1 second heating, a series of condenser discharges was used as for stimulation, but allowed to pass between E and G, fig. 3, *i.e.*, through the lower portion of the nerves lying on the thermopile, without any shunt or series resistance. Since the resistance of the nerves is

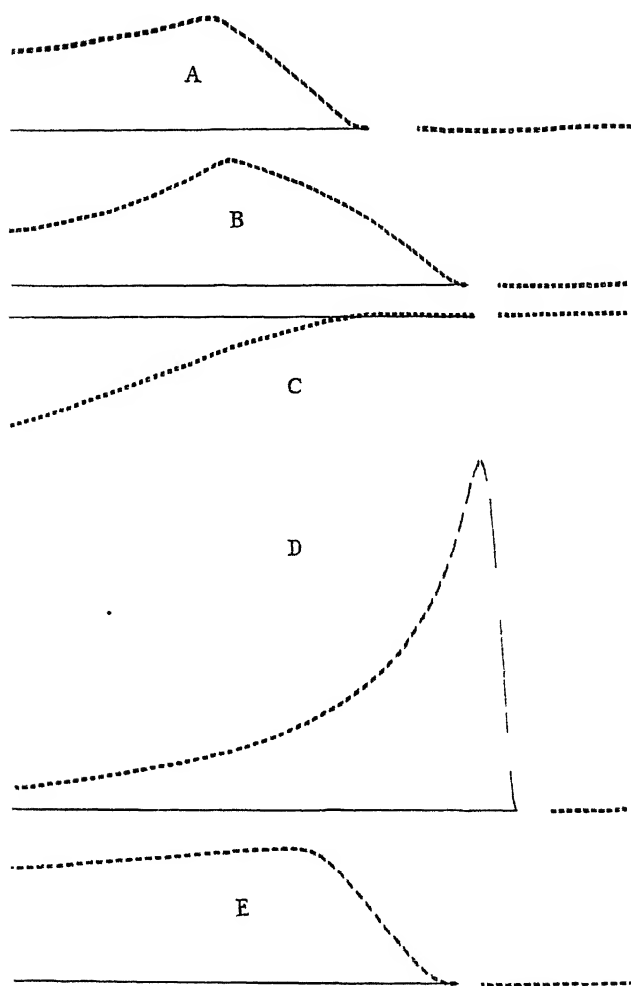


FIG. 7.—Records of nerve heat production at 19°C . January 25-26, 1932. Zc galvanometer without amplification. Time marks (gaps in curves) every 1 second. Preceding a stimulus (or heating) is a longer gap made by placing the hand in the path of the light coming to the camera. Stimulation (or heating) began at the moment the hand was withdrawn which was also at a time signal. The line recorded before stimulation was extrapolated as shown, to serve as a base line for each curve. All curves read from right to left. A, 16 seconds maximal stimulus in oxygen, B, 32 seconds stimulus in oxygen next day. C, record of heat leak, 16 seconds "stimulus" applied to the asphyxiated nerve, the energy in the "stimulus" being about 50 times as great as that of the actual stimuli employed with the live nerve. Heating upwards, cooling downwards. D, 1 second heating control. Below: E, a record of 12 seconds stimulus made after a long rest, in another experiment under similar conditions at about 20°C . Note how the curve stays out, corresponding to greater (or earlier) recovery heat.

high it is necessary to use a rather small capacity and a rather low frequency (see Hill, 1931, *a*, p. 279). Otherwise the energy of the condenser will not be completely discharged and the heat will be too small; the quantity $e^{2t/RC}$ must be negligible, where t is the time allowed for one charge or discharge, R is the resistance in ohms, C the capacity in farads. Putting $R = 3 \times 10^4$, $C = 2 \times 10^{-7}$ ($F = 0.2$ mfd.) and assuming that it is sufficiently accurate to make $e^{2t/RC}$ less than 0.01 we calculate that $2t/RC$ must be greater than 4.6, so that t must be greater than 0.014. Thus not more than 35 charges and 35 discharges can be allowed per second. In practice the commutator was run at about half this speed.

The same short-circuit key was used as for stimulation; exactly at one signal by the metronome the key was opened, exactly at a later one it was closed again; during the interval the discharges passed through the nerves. Tests with a magnet writing on a smoked drum proved that this method of timing was accurate enough. The moment at which the key was pressed and heating commenced was shown, as in stimulating, by withdrawing the hand which cut off the light from the camera.

The commutator, which had a heavy flywheel and ran very steadily, was timed with a stop-watch (a bell marking every 100 turns), the condensers were carefully calibrated against a standard, and the voltage was read with an accurate Weston voltmeter. The energy used in making the control was then

$$5FV^2 \text{ (time of heating) (frequency of charge and discharge).}$$

From this, after the portion of the nerves between the heating electrodes had been weighed, the absolute value of the control curve could be calculated in ergs per gram of nerve. The analysis being in blocks expressed as fractions of the control, the result could be stated at once in ergs per gram per second.

Several control curves were made and measured up at the intervals required. The results were averaged and used for the analysis. In some cases the early part of the control record was made on rapidly moving paper, so as to give greater accuracy at the beginning.

These control curves are rather exact, since the galvanometer can be made less sensitive (and therefore more steady) by reducing the amplification, correspondingly more energy being used for heating. It is one virtue of the system of amplification employed that the dynamical properties of the moving coils are quite unaltered when the sensitivity is changed either by altering the light or by shunting the current from the photoelectric cell. The control

records therefore need not be many since they are far more accurate than those of the "live" heat production.

Allowance for Heat Leak from the Electrodes.—In order to ensure approximately maximal response rather strong stimuli must be employed, and the energy of these cannot be absolutely cut off from the thermopile, particularly when longer stimuli are given. It may be necessary therefore to allow for the heat leaking from the electrodes. This is easily and accurately done by waiting till the nerves are dead, or rendered inexcitable by asphyxiation, and then applying a similar but much stronger "stimulus" and recording the result. For example, with a stimulus of 6 volts and a given capacity, frequency, shunt and series resistance, a certain "live" record is made. When the nerve is inexcitable a "stimulus" of 60 volts is applied with everything else the same, and a "heat leak" curve is recorded. The effect of this is 100 (*i.e.*, $(60/6)^2$) times as great as that of the stimulus to the live nerves. Such a record is shown in fig. 7c. The allowance is generally small, in all the cases of short stimuli discussed here it was quite negligible, but for longer stimuli it may be important, so a ready method of making it is valuable. One great advantage of the method of stimulating by condenser discharges is that their energy is accurately known; such allowances therefore can be exactly made. The heat leak is chiefly a negative one (see fig. 7c), so the correction has to be *added* to the live records.

The Analysis.—In analysis by long intervals (see Hill, 1929, p. 168) it is justifiable to make all the remainders zero. This is always possible, but if one does so when analysing by short intervals small errors in the record to be analysed are apt to set up "oscillations" (see Hartree, 1931) and ridiculous results may be obtained. A balance must be struck between the two criteria:—

- (a) The remainders must be as small as possible throughout; and
- (b) The result must be reasonably smooth.

To insist on making the remainders, particularly the early remainders, zero may lead, either to large remainders later, or to a wildly oscillating analysis. For shorter intervals of analysis more accurate curves are necessary—hence the advantage of photographic recording, and of taking the mean of a large number of records (see fig. 8). With the accuracy at present available, and employing the mean (say) of 50 records, it is just possible to analyse in $\frac{1}{2}$ second intervals, (see figs. 11 and 12). To attempt to work with shorter intervals than this would lead merely to indeterminate results. The only way to satisfy oneself that an analysis is really determinate is to try to find an alternative solution, appreci-

ably different from the first, bearing in mind the two criteria mentioned above. For the sake of those who may wish to be satisfied on this point, the necessary data, and the results of their analysis, for several of the experiments described here, are given in Appendix I. I do not believe that significant deviations from the results given can be obtained without offending one of the criteria. The remainders were always so small that the curves could be reconstructed

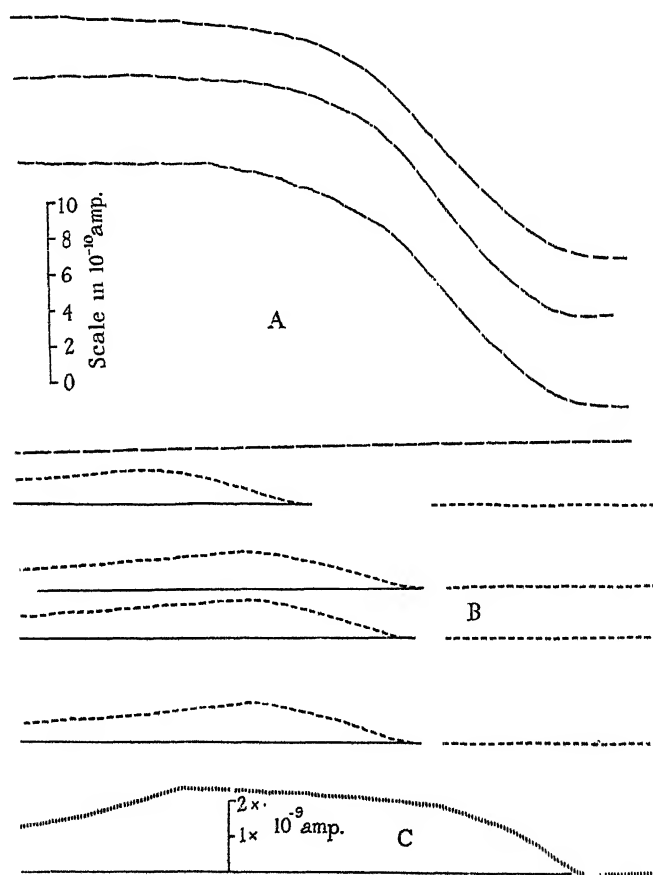


FIG. 8.—(A) *Top*: Experiment of December 2, 1931, at 18.3°C . Records of nerve heat production in three 4-seconds stimuli. Time marks every $\frac{1}{2}$ second. Rapid galvanometer with amplification. 1 mm. on original record = 2.5×10^{-11} amp. For analysis, see fig. 11. (B) *Middle*: Experiment of January 31, 1932, at 0°C . Records of heat production in four 16-seconds stimuli. Time marks every 1 second. Zc galvanometer, no amplification. The stimulus began at the end of the gap in the record. The base line preceding excitation was extrapolated beneath the curve for purposes of measurement. (C) *Bottom*: Experiment of March 8, 1932, at 0°C . Record of heat production in 2 minutes continuous stimulation. Details as in (B). 1 mm. on original record = 1.25×10^{-10} amp. All read from right to left. Heat production upwards.

from the results of the analysis without sensible deviation from the originals.

In many experiments records were made for several durations of stimulus. It is obvious that the records, and their analysis, must be the same for any two durations, up to the moment when the earlier stimulus ends, after that they diverge. It is easier and more accurate to analyse the record of a heat production which changes more slowly, and in which no sharp discontinuity occurs, than of one in which there is a sudden drop in heat rate at the moment when the stimulus ends. Hence, in the analysis of *e.g.*, a 1- or 2-second stimulus, it is an advantage to have made previously the analysis of a longer stimulus, *e.g.*, of 8 seconds. The blocks of heat chosen to represent the 1st and the 2nd second are not determined simply by the galvanometer readings at 1 and at 2 seconds but largely by those later on. Hence, if the heat production occurs continuously for some time after 2 seconds there is more chance of judging correctly what blocks of heat to choose for the first two intervals. Having completed an analysis (say) for 8 seconds it can be assumed that for the shorter stimuli, *e.g.*, of 1 or 2 seconds, the result must be the same up to the end of the stimulus, and the lower heat rate suddenly setting in when the stimulus ends can then be dealt with more effectively on the basis of an assured value of the rates obtained during the last intervals of the stimulus. Several records of such multiple analyses in $\frac{1}{2}$ second intervals are shown in figs. 11 and 12.

In the case of longer stimuli it is extremely laborious to carry out the analysis in short intervals. Usually, therefore, longer intervals have been adopted, actually without loss in the information gathered from them, since the rapid changes at the start and end of a stimulus are best seen in the analyses of comparatively short stimuli. In the longer stimuli, however, the large intervals employed make the shape of the curves of rate of heat production appreciably in error at the beginning and end.

Various tests of the method of analysis were made, of which two are given in Appendix I (E).

It is instructive to reverse the usual process of analysis and to synthesise a deflection for some assumed heat rate during a stimulus. In fig. 9 is a photographic record of nerve heat production for the complex case of 16-seconds stimulus, 8 seconds rest, 16 seconds stimulus, 8 seconds rest, etc. From the equations given at the beginning of this paper, for $A/a = 12\frac{1}{2}$, $B/b = 500$, $a = 1.6$, $b = 0.004$, and an "initial" heat rate during stimulation of $37\frac{1}{2}$ units per second, the total heat rate was calculated for 92 seconds for the same conditions, viz., 16 seconds "on," 8 seconds "off," etc. This was then plotted

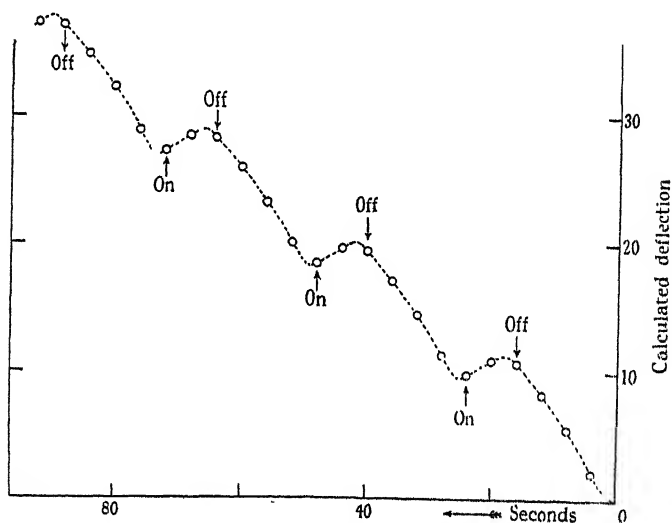
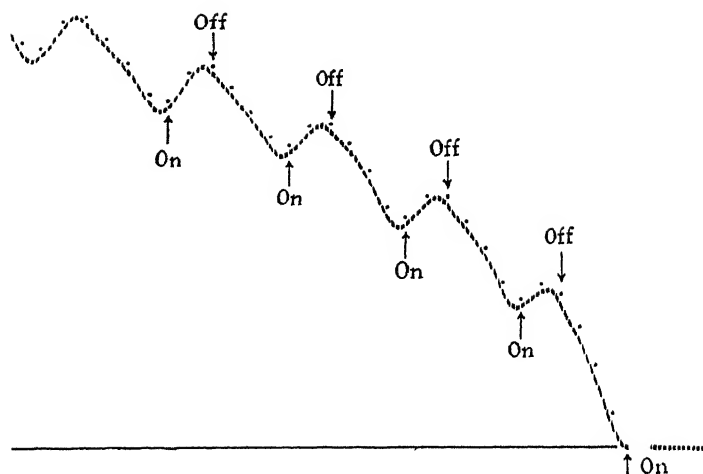


FIG. 9.—Record of nerve heat production at 18.5°C . March 1, 1932. Time marks every 1 second; dot above every fourth time mark. Zc galvanometer without amplification. Stimulus 16 seconds on, 8 seconds off, 16 seconds on, 8 seconds off, etc. Compare with calculated record, fig. 10. Read from right to left.

FIG. 10.—Galvanometer deflection calculated for the case of 16 seconds stimulus, 8 seconds rest, 16 seconds stimulus, 8 seconds rest, etc., for comparison with deflection actually observed (fig. 9). Horizontally, time, read from right to left; vertically, deflection, calculated with a 4-seconds heating control from a rate of heat production calculated as described in the text.

and divided up into 4 seconds blocks as in fig. 1. A 4-seconds heating control had been made during the experiment, as follows :—

Time (seconds)	0	4	8	12	16	20	24	28	32
Deflection	0	412	585	341	260	203	166	140	122

Time (seconds)	36	40	44	48	52	56	60	64	68
Deflection	108	96	87	79	72	67	62	58	55

Time (seconds)	72	76	80	84	88	92	96		
Deflection	53	51	49	47	45	43	41		

With this and the 4-seconds blocks a deflection was calculated which is shown in fig. 10. The calculated 4-seconds points are shown by circles on the curve.

The agreement with fig. 9 is striking; not only is there general similarity but even such a subtle characteristic as a little bend in the curve between the 4th and the 8th second of each step is apparent in both. It is clear that by assuming an initial heat followed at once by a recovery heat of the general type of curve A, fig. 1 (and equation (I)) we can build up deflections exactly like those recorded.

Results.

Short Stimuli at Room Temperature.—The results obtained in short stimuli at room temperature (about 19° C.) are best illustrated by the following examples and by figs. 11 and 12.

Experiment of December 2, 1931.—18·3° C. Ten nerves placed on the thermopile in oxygenated Ringer's fluid on the evening before. Solution removed and replaced by oxygen at 9 a.m. Galvanometer Zd, 3·6 seconds complete period, amplified by photo-cell and Zb galvanometer, 1·9 second period. Photographic records of $\frac{1}{2}$ second (48), 1 second (36), 2 seconds (15) and 4-seconds stimuli (12), taken at intervals of a few minutes throughout the day, interspersed with one another. Two hundred and twenty shocks per second. Typical 4-seconds records in fig. 8. All the records at each duration were measured at every $\frac{1}{2}$ second and averaged, without selection, and the four mean curves so obtained are shown in fig. 11 (upper) without smoothing. After asphyxiation, "instantaneous" heating control curves were made, from which a $\frac{1}{2}$ second heating control was constructed by addition. The analysis of the

mean numbers was then carried out with the results shown in fig. 11 (lower). For details of analyses see Appendix I (A).

The total heat (in 10^{-6} calories) *during* the several stimuli was as follows :—

Stimulus duration (seconds)	$\frac{1}{2}$	1	2	4
Heat (microcalories per gram)	1.35	3.65	9.5	22.7

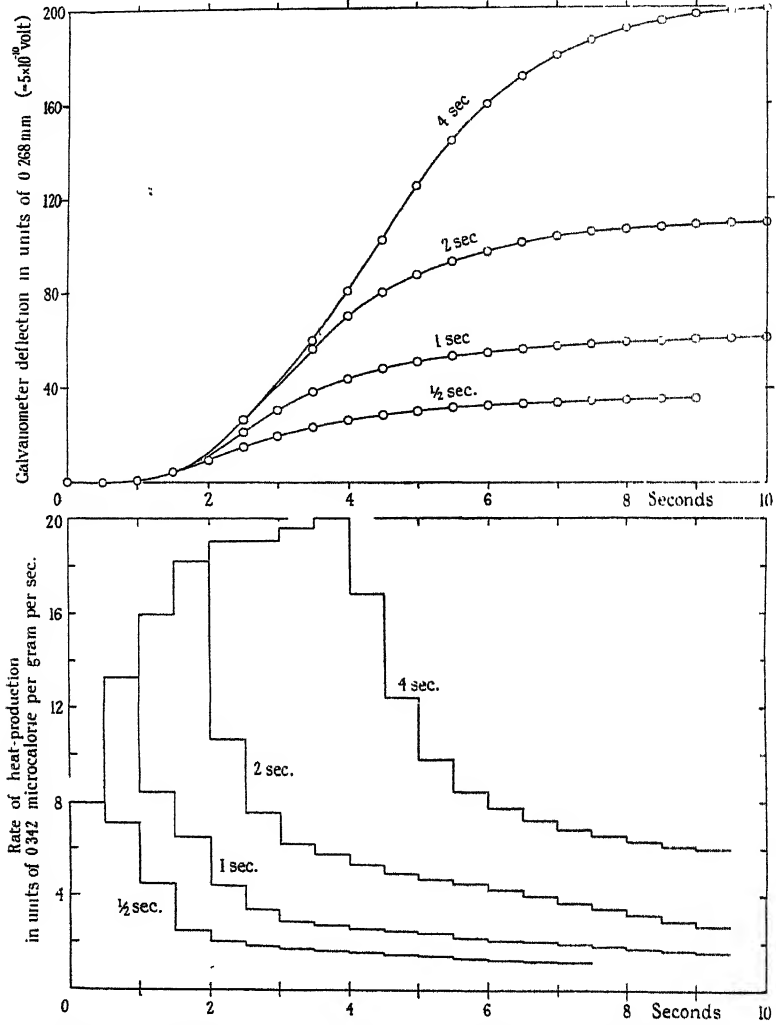


FIG. 11.—Heat production of nerve at 18.3° C. December 2, 1931. $\frac{1}{2}$ -second stimulus, mean of 48 records; 1 second, mean of 36; 2 seconds, mean of 15; 4 seconds, mean of 12 (see fig. 8, top). Nerves used on day following dissection. 220 shocks per second. Rapid galvanometer with amplification. (A) Upper half, actual mean values (circles) of deflection at even $\frac{1}{2}$ seconds, in units of 0.268 mm. (1 mm. = 2.5×10^{-11} amp.). (B) Lower half, results of analysis in $\frac{1}{2}$ second units of 0.171 microcalorie per gram.

The stimuli were nearly maximal, both in strength and in frequency. If we assume the initial heat to appear during a stimulus at a rate not greater than that during the first half second, then of the heat during the 1 second stimulus 26 per cent. is caused by the rapid onset of recovery, of that during the 2-seconds stimulus 43 per cent., of that during the 4 seconds stimulus 52 per cent.

Bronk, working with 3-seconds intervals of analysis at a mean temperature of about 22° C., found the initial heat to be, on the average, about 8·9 per cent. of the whole (up to complete recovery). The time-scale of nerve alters with a change of temperature and 3 seconds at 22° C. is equivalent to about 5 seconds at 18·5° C. The results of the present experiment show that of the heat during the first 3 seconds of Bronk's analysis about 55 per cent. must have been due to recovery, 45 per cent. only being true initial heat. Calculated in this way the initial heat was not 8·9 per cent. but only 4 per cent. of the whole.

Experiment of November 26, 1931.—About 19° C. Details as above. Photographic records of 1 second (53 records), 2 seconds (13), 4 seconds (13) and 8 seconds (12) stimuli. Two hundred and twenty shocks per second. For results see fig. 12 (upper).

The total heat during the several stimuli was as follows :—

Stimulus duration (seconds).	1	2	4	8
Heat (microcalories per gram)	3·3	7·8	19·6	49·0

Assuming the initial heat to be given by the first $\frac{1}{2}$ -second step in the analysis this is 1·5 microcalories per $\frac{1}{2}$ second as compared with 1·35 in the preceding experiment. Thus of the total heat in 1 second stimulation 10 per cent. is due to recovery, in 2 seconds 23 per cent., in 4 seconds 39 per cent., in 8 seconds 51 per cent.

In the lower half of fig. 12 are given :—(A) a curve representing the mean of 53 records of 1-second stimulus ; (B) a curve representing the mean 1-second heating control ; and (C) the analysis of (A) by (B), in 1-second blocks. (C) may be compared with the $\frac{1}{2}$ -second analysis in the upper half of fig. 12. More information as to the form of the heat response is yielded by the $\frac{1}{2}$ -second analysis ; the general form, of course, is the same.

The experiments just described (figs. 11 and 12), and several others giving similar results, were made on nerves prepared about 15 hours before stimulation began, and kept all night in Ringer's fluid on the thermopile in the thermostat. This procedure was adopted for two reasons : (a) in order to have a full day

for the observations, and (b) in the hope of obtaining better thermal and vapour pressure equilibrium by longer soaking.

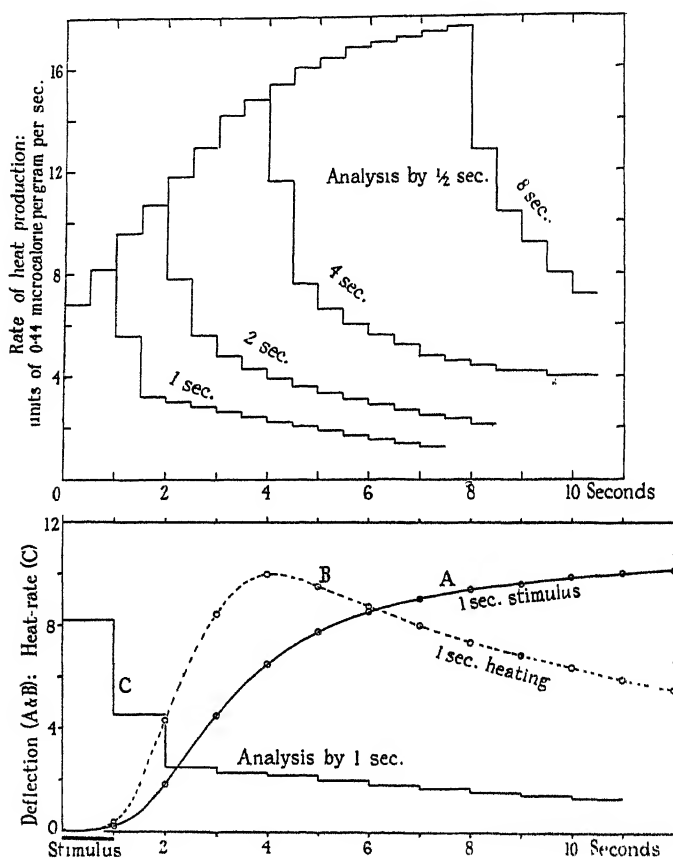


FIG. 12.—Heat production of nerve at 19° C. November 26, 1931. Nerves used on day following dissection. 220 shocks per second. Rapid galvanometer with amplification. *Upper*: Analysis in $\frac{1}{2}$ second units of 0.22 microcalorie per gram. 1 second stimulus, mean of 53 records; 2 seconds, mean of 13; 4 seconds, mean of 13; 8 seconds, mean of 12. *Lower*: (A) 1 second stimulus, mean of 53 records, average height 13.5 mm. at 12 seconds; (B) 1 second heating control. Analysis (C) in 1 second units of 0.445 microcalorie per gram.

It was found later that 2 hours soaking in the thermostat, with vigorous stirring of the Ringer's fluid by bubbling oxygen through it, was enough to give good thermal equilibration. It had originally been intended to measure the resting heat rate of the nerves, for which purpose long soaking (to eliminate vapour pressure differences, see Hill and Kupalov (1930)) is essential. The thermopile, however, was found to be ill-suited to this purpose, owing to its

asymmetry, and the project was dropped. A separate study of the resting heat rate has been made by my colleague, Mrs. M. Beresina, with the symmetrical thermopile described by Downing and Hill (1929), the results of which will be reported separately. Long soaking, therefore, became unnecessary, and it was found that if dissection began at 8.30 a.m. the nerves could be on the thermopile by 10.30 a.m. and observations begun by 1 p.m.

It was then observed that the fresher nerves gave about twice as much heat as those which had been kept all night in Ringer's fluid. In other respects they seemed to be the same. A typical experiment on fresh nerves is shown in fig. 13. Here (A) represents the analysis of the mean of 27 records of 12-seconds stimulus taken at various times between 3 and 7.30 p.m., while (B) is of 8 records of 8-seconds stimulus taken from 7.30 to 8.8 p.m. The agreement between the two, up to the end of the shorter stimulus, is quite good, and shows

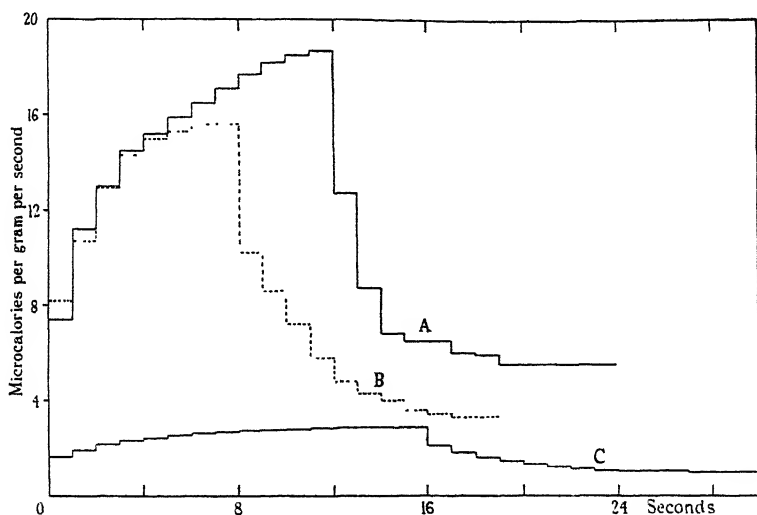


FIG. 13.—Analysis of nerve heat production recorded without amplification by Zc galvanometer. Nerves used (in oxygen) on same day as dissection. Curves (A) and (B): experiment of February 3, 1932, at 21.3°C . Frogs acclimatized to room temperature for some days beforehand. (A), mean of 27 records of 12-seconds stimulus, various frequencies, at 4 to 5 minutes intervals, at various times between 3 and 7.30 p.m. Stimuli mostly not maximal; results multiplied up to represent maximal response. (B), mean of 8 records of 8-seconds maximal stimulus, 450 shocks per second, at 4 to 5 minutes intervals, following the preceding, from 7.30 to 8.8 p.m. Curve (C): experiment of February 9, 1932, at 0°C . Mean of 24 records of 16-seconds stimulus, various frequencies, at various times about 5 minutes apart a few hours after dissection. Results multiplied up to represent maximal response. The three curves are on the same scale, both of time and of heat production. Analyses by 1 second heating control, after allowance (small) for heat leak.

that the nerves were not changing rapidly. Again we see the characteristic rapid rise at the beginning, and rapid fall at the end, of stimulation. Typical records, made with fresh nerves without galvanometer amplification, are given in fig. 7.

These experiments at room temperature, as pointed out above, did not give decisive evidence of the existence of initial heat. It was possible, as in fig. 2, to draw a smooth curve for the heat rate running down to the origin, without exceeding the limits of error of the analysis. The quickest available galvanometer with sufficient sensitivity had been used (3.6 seconds period with amplification) and a very rapid thermopile, and analysis in time units of less than $\frac{1}{2}$ second would be indeterminate. It was impossible to make the instruments quicker; the only possibility was to make the nerves slower, viz., by cooling to 0°C . The objection to this is that they give out much less heat at the lower temperature. This is shown in fig. 13, where (C) is the analysis of records of 16-seconds stimulus at 0°C ., made under conditions strictly comparable with those of the experiment of which (A) and (B) are the result.

Short Stimuli at 0°C .—Typical records obtained at 0°C . without galvanometer amplification are shown in fig. 8. These are very small, much smaller than at 20°C .; compare for example the 16-seconds records at 0°C . in fig. 8 with the 16- and 12-seconds records at about 20°C . in fig. 7. They can however be measured up rather exactly and if sufficient records be taken the mean can be made to yield an accurate analysis.

Many experiments have been performed, of which details of two are given in fig. 14. Both were made on cold-acclimatized frogs as soon as possible after dissection. The records were measured up every 1 second, and the actual means in each experiment are shown by circles; smooth curves (A) have been drawn through these. The analysis in 1 second units shows (i) a sudden rise at the beginning, (ii) a gradual rise during the stimulus, (iii) a sudden fall at the end, and (iv) a gradual fall thereafter. In the experiment of January 8 the heat in the first 1 second was 3.4 microcalories per gram, in that of January 21 it was 2.03 microcalories per gram.

Another analysis of a 16-seconds stimulus at 0°C . is given in fig. 19 below. This agrees with those of fig. 14 in showing that the curve of heat rate during stimulation at 0°C . cannot be supposed to work up gradually from zero, but requires a sudden jump up at the beginning, a sudden jump down at the end. The existence of "initial heat," therefore, as a separate entity is certain.

It is noticeable in every case that the fall at the end is not as great as the rise at the beginning. The response presumably diminishes rapidly during

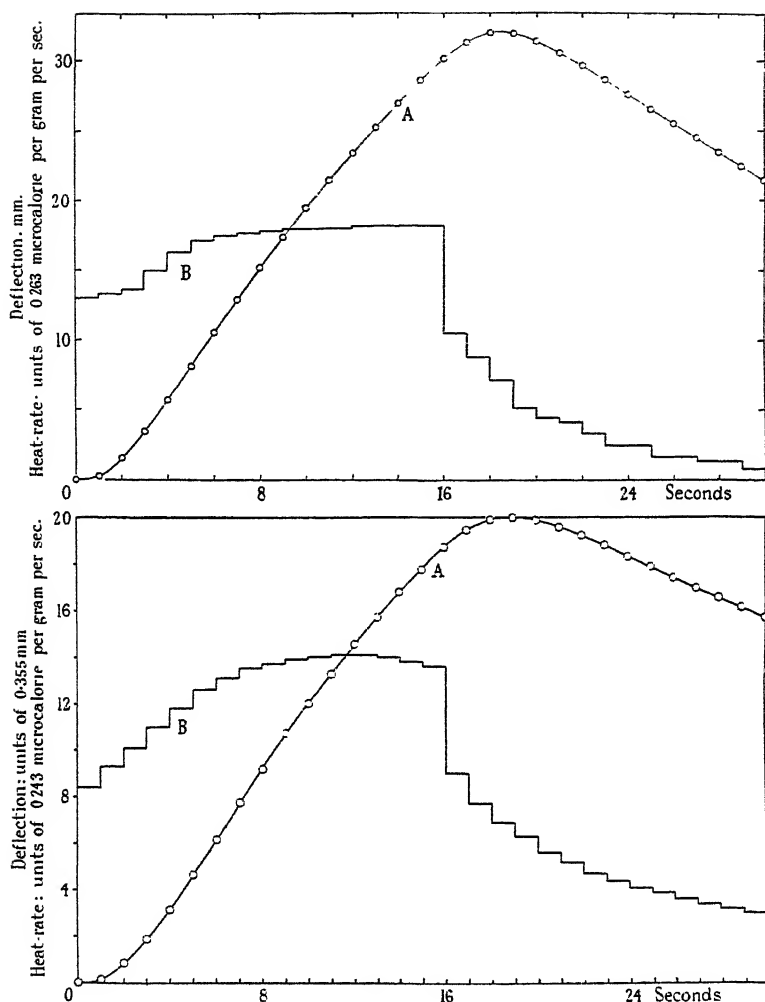


FIG. 14.—Nerve heat production at 0°C . 16-seconds stimulus, analysed by 1 second heating control. Nerves used on same day as, and as soon as possible after, dissection. *Upper*: January 8, 1932. 40 shocks per second. (A), galvanometer deflection, mean of 30 records. Zc galvanometer (3.6-seconds period) with amplification. (B), analysed rate of heat production. For details of the analysis see Appendix I (B), *Lower*: January 21, 1932. 35 shocks per second. (A), galvanometer deflection, mean of 37 records at $5\frac{1}{2}$ minutes intervals. Zc galvanometer (6-seconds period), no amplification. (B) analysed rate of heat production. *Note*.—The stimuli were maximal, but the earliest records were the largest, being about 20 per cent. greater than the mean.

stimulation, at any rate at the relatively high frequency used (30 and 40 per second).

The Initial Heat.—At 0°C. , therefore, the initial heat is a determinate quantity. In figs. 14 and 19, a line drawn through the results of the analysis cuts the vertical axis at a fairly definite point. In nerves from cold-acclimatized frogs examined on the same day as dissection the following four results may be read off from the plotted analyses :—

Date	Jan. 8.	Jan. 21	Feb. 9	March 8	Mean
Initial heat (microcalories per gram)	3.4	1.9	1.5	2.0	2.2

At 20°C. the decision is not so simple. It is clear from figs. 11 and 12 that the average heat rate in the first second may be considerably higher than the initial heat. In fig. 2 the full line drawn through the results of the analyses comes back to 5.7 on the vertical axis, the heat in the first second being 7.3. In fig. 13 a similar extrapolation might give an initial heat of about $5\frac{1}{2}$, the heat in the first second being about 8. It is not possible at present to make a closer analysis. The only thing to do is to take the heat in the first second as the initial heat, realising in so doing that the result may be considerably too great.

The heat in the first second of maximal stimulation of warm-acclimatized frogs on the same day as dissection had the following values in three experiments at about 20°C. :—

Date	Jan. 25.	February 3.		March 1.	Mean.
1st second (microcalories per gram)	7.3	7.4	8.2	8.1	7.75

This is more than three times as great as at 0°C. The difference, however, is entirely due to the fact that cold nerves are unable to respond to a high frequency of stimulation.

The Heat in a Single Isolated Impulse.—The curve of fig. 5 allows us to calculate, from the heat per second in a maximal response at high frequency, the heat in a single isolated impulse. It is impossible to observe the latter directly. Since, however, the origin of zero heat rate at zero frequency must lie upon the curve, and since the form of the curve is fairly definite a little way from the origin, its shape at the origin can be fairly accurately interpolated. By laying off a tangent at the origin the following results can be calculated for the ratio (heat per impulse at zero frequency) : (heat per second at high frequency),

viz., at 0°C. (cold frogs), 0.12 ; at 21.3°C. (warm frogs), 0.0086.

These results may be expressed in another way: at 0° C. the nerves of cold-acclimatized frogs would give in $8\frac{1}{2}$ isolated impulses, at 21.3° C. those of warm-acclimatized frogs would give in 116 isolated impulses, as much heat as they would in 1 second of stimulation at maximal frequency.

From this we may calculate the heat in a maximal isolated impulse. We will deal with the case of the initial heat only, since the ratio of total heat to true initial heat is not as yet accurately known. In nerves examined on the same day as dissection, and subjected to a maximal stimulus, we have just seen that the "initial" heat is as follows:—

At 0° C. 2.2 microcalories per gram.

At 20° C. 7.75 microcalories per gram.

Thus in single impulses the "initial" heat should be:—

At 0° C. $2.2 \times 0.12 = 0.26$ microcalories per gram.

At 20° C. $7.75 \times 0.0086 = 0.067$ microcalories per gram.

Thus at 0° C. the single impulse gives four times as much heat as at 20° C. The ratio may be even greater, since at 20° C., as we have seen, the true initial heat is probably appreciably less than the total heat in the first second of stimulation. The effect is similar to, but greater than, that found with muscle. Hartree and Hill (1921, pp. 138–141) explained the effect of a fall of temperature in increasing the heat production following a single shock by supposing that the "change of permeability caused by a single shock lasts longer at the lower temperature (as does the electric response) and allows more" chemical reaction to occur "and so more heat to appear." The same hypothesis would apply to the case of nerve. The greater duration of the electric response, and of the chemical reactions presumably underlying it, is responsible for the greater amount of energy set free.

The absolute value of the "initial" heat per isolated impulse at 20° C., viz., 0.067 microcalorie per gram, is less than the value found by Gerard, Hill and Zotterman (1927), viz., 0.1 microcalorie per gram. This might be due partly to the fact that different frogs were used (Hungarian *R. esc.* in the present, Dutch *R. esc.* in the earlier work), but it is sufficiently accounted for by the fact that the initial heat (owing to the improved analysis) is now a much smaller fraction of the whole.

The Source of the Heat.—The initial heat in a single isolated maximal impulse at 0° C., viz., 2.6×10^{-7} calorie per gram, is less than one ten-thousandth part of that in a single maximal muscle twitch; the "initial heat" in a similar

impulse at 20° C., 6.7×10^{-8} calorie per gram, is about one fifty-thousandth part. In the electric change of a single nerve impulse at 6.2° C. (according to the assumptions made (Hill, 1921)) the energy is of the order of 3.5×10^{-10} calorie per gram, about 1/500 of that in the impulse itself (interpolated for 6.2° C.).

Presumably some chemical reaction is responsible for the initial heat. The mere "mixing" of electrolytes would not explain it. The breakdown of phosphagen may be assumed to liberate 120 calories per gram of H_3PO_4 . According to Gerard and Tupikow (1930) about 4 mg. of labile phosphorus per 100 grams of nerve is broken down in asphyxia; reckoned as H_3PO_4 this is about 12 mg.; assuming the labile phosphorus to be in the form of phosphagen this would liberate $12 \times 10^{-5} \times 120 = 1.44 \times 10^{-2}$ calorie per gram, enough for the initial heat in 50,000 isolated impulses at 0° C., for 220,000 such impulses at 20° C. Assuming maximal continued stimulation to liberate initial heat at the rate of 5×10^{-6} calorie per gram per second, the available phosphagen, even if no recovery occurred, would last for $1.44 \times 10^{-2} / 5 \times 10^{-6} = 2880$ seconds = 48 minutes. Actually recovery proceeds so rapidly that only a small fraction of the whole would be unrestored at any moment, even during maximal prolonged stimulation.

According to Gerard and Wallen (1929) "nerves stimulated in an oxygen atmosphere showed an increase in inorganic phosphate at the expense of the combined, when compared with equivalent nerves examined fresh or after an equal period in oxygen. This breakdown of combined phosphate was less than the maximum obtained in asphyxia." It would be well to repeat at 0° C. their stimulation experiments, both in oxygen and in nitrogen; at that temperature the maximum rate of liberation of initial heat is not very greatly reduced, while that of recovery heat is. By diminishing the rate of recovery more than that of the initial breakdown larger differences might be produced,* and there would be more hope of finding directly whether phosphagen is, or is not, the source of the initial heat. The same applies to the other phosphorus compounds possibly responsible for the energy.

A similar calculation can be made for the case of lactic acid. According to Holmes, Gerard and Solomon (1930) the lactic acid content of stimulated frogs' nerves showed a slight and perhaps significant rise, on the average about 5 mg./100 gram, as the result of continuous stimulation for some hours. Assuming 300 cal. to be set free for 1 gram of lactic acid formed and neutralised, their

* One possible objection to this is that nerves "fatigue" much more rapidly at 0° C., at any rate with higher frequency of stimulation.

average value corresponds to $5 \times 10^{-5} \times 300 = 1.5 \times 10^{-2}$ cal. per gram. This, at 5×10^{-6} cal. per gram per second, would last for 3000 seconds = 50 minutes.

The initial heat indeed is so small that chemical changes below the range of possible measurement might be responsible for it. With recovery going on all the time, presumably removing the substances set free, the total amount of these present at any moment must be such that the detection of a difference caused by stimulation, far more the measurement of a difference, presents most formidable difficulties. It is possible to attribute the initial heat either to phosphagen breakdown, or to lactic acid formation, without demanding an amount of chemical change even as great as Gerard and his colleagues have found. Unless the experiments suggested at 0° C. afford a decision, it is difficult at present to see how any direct method can solve the problem of the origin of the initial heat in nerve.

If lactic acid formation, or phosphagen breakdown, be the source of the initial heat, the analogy of muscle affords no basis for supposing that the recovery heat represents merely the oxidative removal of the breakdown products; it is much too large. If recovery heat be, as in muscle, about equal to initial heat, then 95 per cent. or so of it remains to be explained. It seems more likely that the recovery heat, as was suggested by the work of Levin (1927), Furusawa (1929) and Hill (1929, p. 173) on crustacean nerve, is chiefly due to the combustions in which the free energy is liberated by which the nerve is "recharged."

The usual picture of the nerve impulse involves a change of permeability, transmitted from point to point by the action current associated with it; the current running from an active spot to a neighbouring inactive spot somehow renders the second spot active. The local "alteration of permeability" and its rapid reversal cannot conceivably be effected without energy loss; in a system like a nerve any actual change must involve processes which are "irreversible" in the thermodynamic sense, processes which can be reversed only if free energy be supplied by chemical breakdown. The initial heat may be a sign of that breakdown.

On this view a change of permeability at any point is induced by the trigger action of the current flowing from a neighbouring point. Rise and fall of permeability both require energy, just as do rise and fall of tension in an isometric muscle twitch. The change of permeability, however, results in a diffusion of ions and the production of an electric current. The former must be reversed if activity is to be maintained. A kind of secretory process there-

fore goes on, by which the initial ionic condition is restored : in this secretion oxygen is used, combustion occurs, and the recovery heat is set free.

Prolonged Stimulation.—If stimulation (at about 20° C.) be prolonged beyond the 8 or 12 seconds of which analyses have already been given, the rate of heat production continues to increase until an apparently steady state is reached at about 12 minutes. For the analysis of this phase a correction for heat leak from the stimulating electrodes is essential ; the correction, however, can be accurately made, provided condenser stimulation be employed.

To obtain a true picture of the rapid rise and fall of heat rate at the beginning and end of stimulation it is necessary to analyse in short time-units, *e.g.*, of $\frac{1}{2}$ second or 1 second. It would be very laborious, and quite unnecessary, to continue to use such short intervals throughout prolonged stimuli. Longer intervals are sufficient, *e.g.*, of 0.1, 0.2, or even 0.4 minute, provided it be understood that the sudden changes at the beginning and end of stimulation require a finer analysis, that in fact the initial heat and the very early stages of recovery are “lumped together” even when the shortest of these intervals is used. The slower changes, however, are actually better dealt with by longer intervals of analysis, and these have the advantage that the arithmetic is purely mechanical, the remainders being made zero throughout.

In fig. 15 are two curves (on different scales of time) obtained from the same experiment at room temperature, (A) of 4 minutes and (B) of 20 minutes stimulation. Details are given in the legend. The nerves had completely recovered from previous excitation before each stimulus. An approximately steady state was reached in about 12 minutes. It is possible that this steady state is apparent and not real, a diminishing initial heat rate balancing a recovery heat rate still increasing towards its maximum. If it be real,* then initial heat and recovery heat must be occurring at corresponding rates, and we have what is probably the best method of finding the value of the ratio (initial heat)/(total heat). The total heat rate is given by the constant deflection corrected for heat leak, the initial heat rate can be determined by omitting a short interval of stimulation and analysing out the sudden decrease in heat rate from the negative deflection so obtained.

It is hoped to apply this method to a more accurate study of the (initial heat)/(total heat) ratio. Fig. 15 gives an indication of the result. From the analyses given above it is

* Recent experiments by my colleagues Beresina and Feng show that a genuine steady state is attained if the frequency of stimulation is not too high ; with higher frequencies the heat rate reaches a maximum and then declines. There are signs of this in fig. 15 (B).

clear that the average heat rate during 24 seconds stimulation must be quite $3\frac{1}{2}$ times as great as the heat rate during the first second, so that the initial heat rate at the beginning of curve B, fig. 15, must have been about 2.5 microcalories per gram per second. The nerves were being used on the day following dissection, and under such conditions the heat rate in the first second has been found in two other experiments with maximal stimulation to have the values 3.4 and 3.3; in the submaximal stimulation of fig. 15, 2.5 is a reasonable value. The total heat rate at the top of curve (B) is about 39. Assuming that the initial heat rate at this stage is still 2.5 the ratio (initial heat)/(total heat) = 1/16. The "initial heat" can be found directly by omitting a short interval of stimulation and analysing the record so obtained: this it is intended to do.

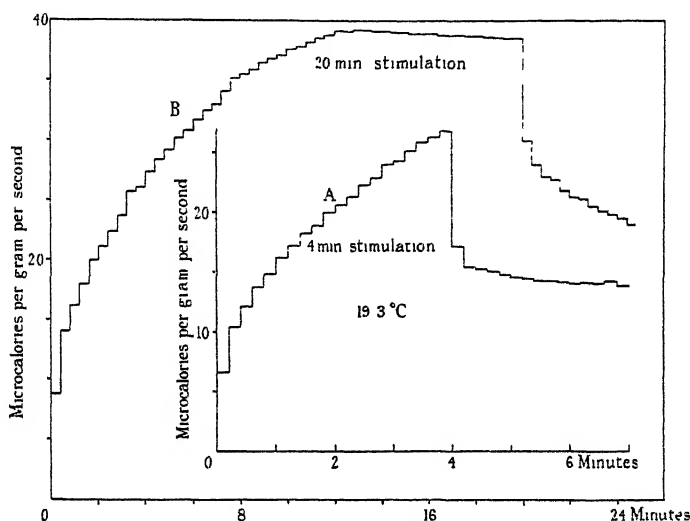


FIG. 15.—Prolonged stimulation. Analysis of data obtained by reading galvanometer deflection on scale. Experiment of November 5–6, 1931. 19.3° C. Nerves used on second day, about 18 hours after dissection. Stimuli 140 shocks per second, not maximal either in strength or frequency, to avoid excessive heat leak correction. Zc galvanometer, no amplification. (A) mean of 3 records, in good agreement, of 4-minutes stimulation; 1 hour recovery after each record. Analysis in 0.2 minute units by 0.2 minute heating control. (B) single record of 20-minutes stimulation, analysed in 0.4 minute units. (The maximum deflection on the scale was 113 mm. at a sensitivity of 10^{-6} volt = 49 mm.) Note.—In each analysis the first block does not represent the true initial heat but contains a large amount of recovery heat, as shown in other experiments by analysis in short intervals. Judging from those other analyses the true initial heat in each case must have been about 3 microcalories per gram per second.

Several experiments have been made at 0° C. with 2 minutes stimulation and 6 seconds intervals of analysis. The results of one of these, with nerves in oxygen, are given in fig. 16 (A). Fig. 16 (B) shows another experiment with nerves in nitrogen. They had been in nitrogen (freed from oxygen by passing

over heated copper) for 15 hours. It was somewhat surprising that the nerves were so good after so long a period of oxygen want: the fact, however, has been confirmed on other occasions. The metabolism at 0°C ., presumably, is so low that it requires more than 15 hours to produce the asphyxiation which is complete in 2 hours at 20°C . This is a further sign that it is not the loss of

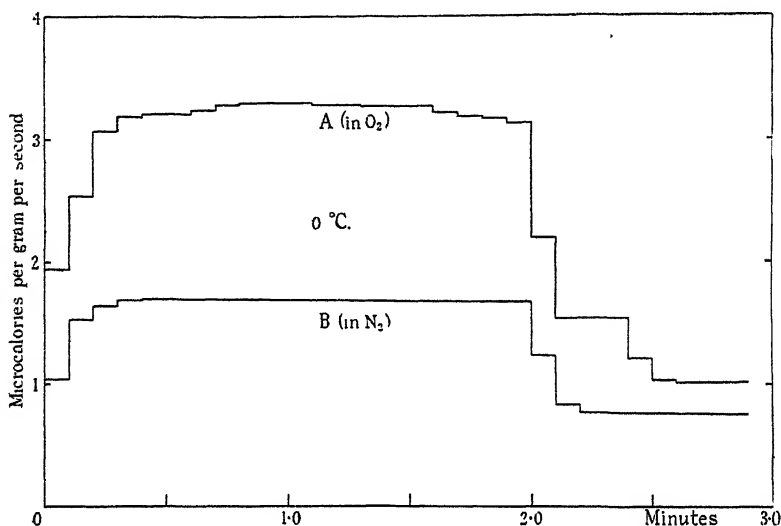


FIG. 16.—Nerve heat production during and after 2 minutes stimulation at 0°C . Analysis in 0.1 minute units, by 0.1 minute heating control, of the mean of five records (see fig. 8 (C)), due allowance for heat leak from the stimulating electrodes. *Upper* (A), experiment of March 8, 1932, 26 shocks per second, nerves in oxygen on the same day as dissection. *Lower* (B), experiment of February 10, 1932, 24 hours after dissection, 23 shocks per second, nerves in pure nitrogen during and for 15 hours before the observations.

molecular oxygen by diffusion which causes the disappearance of excitability but, probably, the exhaustion of some substance potentially available for oxidation.

Curves A (in O_2) and B (after 15 hours without O_2) are strikingly similar: B indeed is only half as high as A, but its shape is almost exactly the same. Both are completely different from those of fig. 15 (prolonged stimulation at 19.3°C). Recovery heat there evidently is, as shown by the rise of the curve at the beginning and its fall at the end of stimulation. The initial rate of rise, however, is not maintained and this is the more striking when we reflect that in some respects the scale of time at 0°C . is only about one-tenth of that at 20°C ., so that 120 seconds at the former is equivalent to about 12 seconds only at the latter; in a 12-seconds stimulus at 20°C . the heat rate is rising very

rapidly at the end. In fig. 16 moreover, the drop in heat rate at the end is much less than the rise at the beginning, indicating that the initial heat largely decreased during 2 minutes stimulation. The same conclusion can be drawn from all the shorter analyses, made with 1 second intervals, at 0° C. This rapid decrease of the initial heat (and therefore of the recovery process following it) is the reason why the rate of heat production at 0° C. so soon reaches a maximum and then begins slowly to decline.

This early "fatigue" of nerves stimulated about 30 times per second at 0° C. has been confirmed by special experiments in which gaps of 16 seconds have been interposed during otherwise continual stimulation. The negative deflections so caused measure the response at the moments in question: they may be compared with one another and with the positive deflection to a stimulus of 16 seconds in a previously resting nerve. The response diminishes rapidly at first, and then more slowly, tending to reach a constant small value. There is a similar, but much smaller, decrease in the response at 20° C.; at the higher temperature the recovery heat rate builds up at first so rapidly, and the response declines so slowly, that (as shown in fig. 15) many minutes are needed for the "fatigue" effect to become visible.

The calculation given above (p. 108) with the same constants, can be extended to cover the case of 20 minutes continuous stimulation (fig. 17 *cf.* with fig. 15).

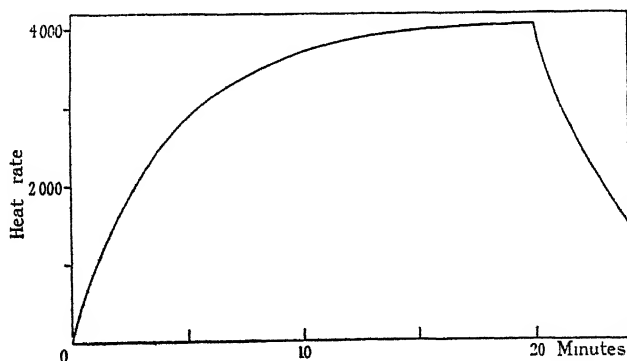


FIG. 17.—Calculated rate of heat production of nerve during 20 minutes continuous "stimulation." No initial heat is included, its inclusion would merely raise the curve very slightly during the stimulus. Note that the curve is very similar to fig. 15 except that the recovery heat diminishes more rapidly at the end.

The steady state is nearly attained in about 15 minutes. The only important difference is that the recovery heat rate falls more rapidly in the calculated than in the observed curve. This difference could easily be avoided by taking

smaller values for the constants b and B . If we did so the calculated curve would attain a steady value more slowly. It is probable indeed that the apparent steady value of the observed curve at about 12 minutes is really due to a balance between a slowly diminishing response and recovery still increasing, and that if a really constant response were given by the nerve the observed curve would go on rising longer.

At 0°C . similar conditions exist. Here recovery is very much slower—the constants a and b would both be much less. An apparently steady heat rate is attained in about 2 minutes, (see fig. 16). The recovery process being slower at 0°C ., a really steady state should be reached more slowly, not more quickly, than at 20°C . The condition is in fact not even approximately steady—the constant heat rate represents a temporary balance between recovery rising and response falling. Equations could be given for such a case, but no purpose would be served as the matter is sufficiently clear.

Oxygen Want.—It was shown by Gerard (1927, *b*) that when oxygen is removed from a nerve at room temperature its heat production falls in absolute amount, finally reaching zero, but the time-course of its evolution remains approximately the same. Recovery heat continues, more or less unchanged in relation to initial heat, so long as the nerve responds to stimulation. Presumably some oxidising reserve is present.

This matter will not be considered here in detail, but various observations taken incidentally in other experiments confirm Gerard's conclusions. In fig. 18 for example, curve (2) represents the analysis of the heat (after due allowance for heat leak from the electrodes) in a 32-seconds stimulus in oxygen at 19°C . Curve (3) is the same after 30 to 60 minutes in nitrogen; curve (4) after 2 hours in nitrogen. Curves (3) and (4) differ greatly in height from curve (2), but very little in shape.

Fig. 19, B, for nerves without oxygen at 0°C . for 15 hours, shows obvious recovery heat. A comparison of this with fig. 19, A, for the same nerves in oxygen at the same temperature, proves that there is practically no difference in the character, as distinguished from the size, of the response, due to deprivation of oxygen, even for 15 hours at 0°C .

The Summation of Responses.—During the analysis of the many records referred to above the question arose whether the responses were additive, in the sense, for example, that the record for 4 seconds stimulation might be deduced by adding two records for 2 seconds stimulation, one starting at time 0, the other at time 2 seconds. Were the response of the nerve constant throughout stimulation, and were the recovery process from later elements of the stimulus unaffected by the recovery process, already going on, from earlier elements, then such would be the case. We have assumed in the calculation on p. 108

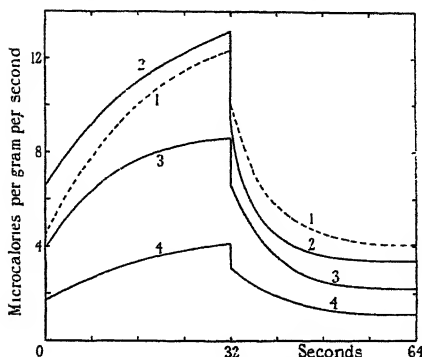


FIG. 18.—Nerve heat production at 19° C. January 26, 1932. 32-seconds stimuli, 156 shocks per second. Records analysed by 4 seconds heating control and smooth curves drawn through the results. The experiment began nearly 24 hours after dissection, the nerves having been in oxygen for about 20 hours. They had been used for the experiment on the previous day. Zc galvanometer without amplification; due allowance for heat leak. Curve 1: analysis of first record made, the nerve being fully recovered having been at rest for more than 12 hours. Curve 2: analysis of the mean of the next 5 records (see fig. 7 (B)) at about 5 minutes intervals. Curve 3: analysis of the mean of 7 records, the nerves having been in nitrogen for 30 to 60 minutes. Curve 4: analysis of the mean of 6 records, the nerves having been in nitrogen for 2 hours.

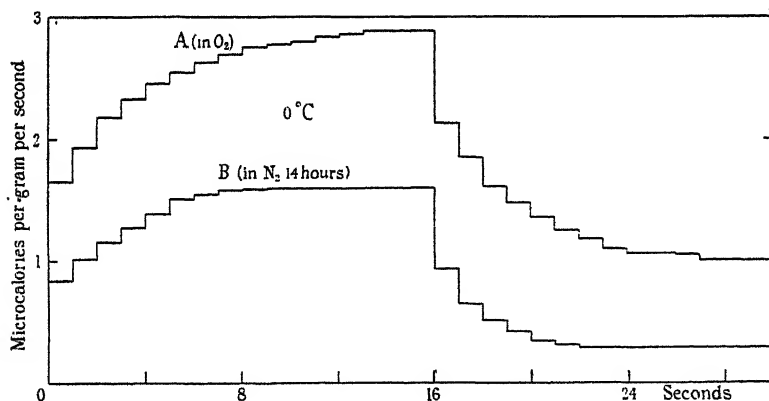


FIG. 19.—Nerve heat production at 0° C. Experiment of February 9–10, 1932. Cold-acclimatized frogs. Upper curve: analysis by 1 second heating control of the mean of 24 records of 16-seconds stimulus in oxygen, various frequencies, taken at various times on the same day as dissection and a few hours after it. Results multiplied up in the ratio (mean height of greatest 6 curves) : (mean height of all 24 curves) = 56/52 to express the heat production of a maximal response. Lower curve: analysis of similar records made next day after about 14 hours in pure nitrogen. 25 shocks per second. Mean of 3, all maximal. Note that after 14 hours in nitrogen the heat production is smaller than, but the shape of the curve is almost indistinguishable from, that obtained on the previous day in oxygen. Heat leak (small) allowed for. Zc galvanometer without amplification. For analysis see Appendix I (D).

above that this is so. In many physical processes such a relation exists; for example, with the recording system used in the present experiments, the curve of response to any liberation of heat may be added numerically to that of any other, to obtain the response when both occur together, or in succession.

It seemed at first very likely that the heat response was additive in this way; the records for longer stimuli could be built up approximately by successive addition from those for shorter stimuli. One complication, however, was soon found to exist, viz., that (at any rate with high-frequency stimulation) successive elements in the addition had to be made progressively smaller: apparently (*e.g.*) the response to the second 2-seconds stimulus, during continuous stimulation, was less than that to the first: the response to the third less than that to the second, and so on. Even so, however, if successive elements of response were of the same "shape," even though of different size, it would be possible to analyse the response to the longer stimulus in terms of that to the shorter, and so to follow nerve activity throughout a long stimulus in a way not otherwise possible.

Further investigation, unfortunately, showed that so simple a relation does not, in fact, exist, except approximately. Attempts to analyse the response to a longer stimulus in terms of that to a shorter led to impossible remainders later on. A special experiment was devoted to the question, in which ten 8 seconds and twelve 32 seconds stimuli were given, the two durations being interspersed with one another so that their results were strictly comparable. The means, and an attempt at analysing the 32 seconds curve in terms of the 8-seconds curve are given below:—

Time (seconds):	0	4	8	12	16	20	24	28
Deflection—								
32 seconds stimulation	0	102	308	485	636	767	870	956
8 seconds stimulation	0	102	309	402	384½	360	336	304
Result	1.0		0.813		0.715		0.589	
Remainder	0	0	-1	0	½	7	0	11

Time (seconds):	32	36	40	44	48
Deflection—					
32 seconds stimulation	1025	988	877	775	689
8 seconds stimulation	295	277½	263	245	233
Result	—	—	—	—	—
Remainder	0	-32	-92	-125	-167

There is no doubt that successive elements in the response to continuous stimulation do in fact diminish in size, as the results of this analysis show. They alter, however, also in shape as the large and increasing negative remainders prove. The heat response of a nerve, therefore, is not an additive quantity; it is affected not only in size but in time-course by preceding stimulation.

This effect of preceding activity is well shown by the difference between a record (see fig. 7 (E)) taken after a very long rest and one taken during a regular series, every 3 or 4 minutes. The recovery heat sets in much more rapidly in the former. Compare also curves (1) and (2), fig. 18. Similarly, one may imagine, recovery from the last three 8-seconds intervals of stimulation in the 32-seconds stimulus referred to above occurs

progressively more slowly than that from the first 8 seconds; consequently, in analysis by the 8 seconds record, large negative remainders are left during recovery.

An effect of previous stimulation on the recovery heat occurs also in muscle, but there in the opposite sense. According to Hartree and Hill (1922), confirmed by many later experiments (some not yet published) by Hartree, the recovery heat occurs at a relatively greater rate after longer stimuli. In nerve the rate is relatively *less* after a longer stimulus. It is not possible, at present, to suggest any cause for the phenomenon.

Discussion.

It is clear, from the experiments at 0° C., that the initial heat really does exist, though its magnitude at 20° C. is uncertain. It is clear also that the recovery has an early rapid phase, complete in a few seconds, and a later slow phase taking half an hour or more. The results of all analyses agree in showing a quick decline in the heat rate during the first few seconds after stimulation ends, followed by a slow decline thereafter. The recovery heat rate can be expressed by a double exponential (equation (I) above)

$$y = Ae^{-at} + Be^{-bt}.$$

A and a refer to the rapid process, B and b to the slow one. The total energy liberated in the A process is far less than in the B process; with the constants given above A/a (which is proportional to the energy in the former) is 100, while B/b (representing that in the latter) is 4000. The second is 40 times the first. The ratio may not be correct, but it is probably of the right order of quantities. If so the total energy in the A process is only a small fraction of the whole, probably of the same order of size as the initial heat.

It is tempting therefore to take the analogy of muscle and to suppose that the initial heat is a sign of a chemical process, *e.g.*, lactic acid formation or phosphagen breakdown, of which the A process in recovery represents the reversal. From the calculation given above (p. 146) there is clearly nothing to prevent us from assuming that either of these reactions occurs to the extent required. We might go further on the analogy of muscle, and suppose that the initial heat is a sign of phosphagen breakdown, the A recovery heat of lactic acid formation supplying energy by which the phosphagen breakdown is reversed. It will be interesting to see whether the A process is abolished by iodoacetic acid.

We are left, however, with the B recovery process, which is of much greater extent. Here the analogy of muscle does not serve. In crustacean nerve this phase of recovery is associated with the restoration of the electrical condition of the nerve, as measured by the size of its demarcation current. In frog's

nerve Gerard (1930) has found that (as in crab's nerve) asphyxia causes a diminution in the demarcation current, which rises again when oxygen is readmitted. The potential difference of which the demarcation current is a sign is perhaps determined by a difference of ionic concentration across some interface. It is natural therefore to connect the B recovery process with the restoration of this ionic concentration difference. Such a restoration is fundamentally of the same nature as "secretion," which we know is rather expensive in energy.

On this view there is no reason why there should be any fixed relation between initial and recovery heat in nerve. The former and the A process of the latter may well be related; they may represent respectively the breakdown, and recovery from the breakdown, supplying energy for the pulse of altered permeability which is supposed to underlie the propagated disturbance. The amount of ionic interchange which occurred as a result of the temporary alteration of permeability would depend upon various factors, but scarcely upon the energy required to transmit the pulse itself. The difference between the nerves of frog and crab, in respect of the ratio (initial heat) : (total heat), would thus become intelligible, and we are encouraged to look for alterations in the ratio, due to changes in the conditions to which nerves are subjected, *e.g.*, temperature or duration of stimulus.

So far as the present results throw light directly on the ratio (initial heat) : (total heat) they prove that previous estimates are too high. The maximum deflection in the experiments by Downing, Gerard and Hill must have measured approximately the total heat in the first 10 seconds of stimulation; reference to the various analyses given above shows that of the heat during 10 seconds (at 20° C.) at least half is recovery heat. They found the initial heat (measured by the maximum deflection) to be 11 per cent. of the whole. This should be corrected, therefore, to 5 per cent. We have referred above (p. 139) to Bronk's experiments; taking account of recovery heat coming on during the first 3 seconds his value of the ratio should be reduced to 4 per cent. This is still twice as great as in crabs' nerves, and in these a closer analysis will probably reduce the ratio below the present estimate of 2 per cent.

[*Added in proof.*—One possible explanation of the initial heat is that it is derived from the energy of a condenser discharged during the transmission of the impulse. On this hypothesis an electrical double layer existing at some cylindrical surface in the fibre is momentarily discharged during activity and rapidly reformed. The potential difference across it is naturally assumed to be that of the "resting" or "injury" potential, say, 50 mv.

Suppose that the double layer considered is located at the surface of the fibres, and (for the sake of calculation) that one half of the nerve is composed of fibres, of mean diameter 10μ . The total area then is 2000 sq. cm. per gram of nerve. In *Valonia* and *Nitella* Blinks (1929) has shown that the capacity of the surface is from 0.1 to 1 mfd. per sq. cm. In red blood corpuscles Fricke (1927) has found a capacity of 0.8 mfd. per sq. cm. Taking 0.8 mfd. as a possible value for nerve, the total capacity per gram would be 1600 mfd. Charged to 50 mv. this would have energy $5 FV^2 = 20$ ergs = 0.48 microcalorie per gram. We have found 0.26 microcalorie at 0°C ., 0.067 microcalorie at 20°C ., for the initial heat in a single isolated impulse.

Calculated roughly in this way the energy is of the right order of size, rather too large at 0°C ., seven times too large at 20°C . The condenser, however, need not be fully discharged: the difference, in fact, between low and high temperature might be that at high temperature the time for discharge and the degree of discharge are less.

The capacity assumed, 0.8 mfd. per sq. cm., corresponds, with a dielectric constant of 4, to a condenser with parallel plates 44×10^{-8} cm. apart. This represents a film one, or a few, organic molecules thick. Thus the discharge, or the partial discharge, of a condenser distributed along the nerve fibre, assuming perfectly reasonable values for potential and capacity, would give us the required initial heat.]

Summary.

(1) The heat production of stimulated frogs' nerves has been reinvestigated with improved methods, including (i) a more sensitive and quicker thermopile, (ii) galvanometer amplification by photoelectric cell, (iii) condenser stimulation, and (iv) photographic recording.

(2) It has proved possible to record the heat in stimuli as short as $\frac{1}{2}$ second (at 20°C .), and to analyse it in $\frac{1}{2}$ second intervals.

(3) At 20°C . the rate of heat production rises rapidly at first during stimulation and falls rapidly at first when stimulation ends. Experiments at this temperature provided no decisive evidence of the existence of "initial" heat; the whole heat might have been due to "recovery."

(4) At 0°C . the heat production is less, but can still be recorded and analysed. The processes in nerve are so much slower at 0°C . that the analysis is much more effective; it provides decisive evidence that "initial" heat exists as a separate entity.

(5) In maximal stimulation of a fresh nerve the initial heat at 0° C. is about 2 microcalories (2×10^{-6} cal.) per gram per second; at 20° C. no exact value can be given, but it is not greater than the heat rate in the first second, viz., about 8 microcalories per gram per second.

(6) The effect on the heat production of frequency of stimulation has been reinvestigated at two temperatures. At 0° C. a maximum response occurs already at 30 shocks per second; at 20° C. not below 400 per second. The smaller heat in stimulating at a low temperature is due solely to the lower frequency of possible response.

(7) The "initial" heat in a single isolated impulse at 0° C. is 0.26 microcalorie per gram; at 20° C. it is not greater than 0.067 microcalorie per gram. The greater heat at the lower temperature is probably connected, as in muscle, with the greater duration of the single response.

(8) For a given energy in the stimulus, the heat response of a nerve varies with the time of discharge of the condenser supplying it; if F microfarad be the capacity employed and R the resistance in series with it, the heat is a function of RF . The "optimal" stimulus at about 20° C. is for $RF = 160$, at 0° C. for $RF = 900$. These represent times of half discharge of 0.11 σ and 0.58 σ respectively.

(9) In prolonged stimulation at 20° C. the rate of heat production goes on rising for a long time until an approximately steady state is reached. Recovery is not complete for an hour.

(10) In prolonged stimulation at 0° C. the rate of heat production soon reaches a maximum; there is no steady state, the response diminishes as stimulation continues.

(11) The absence of oxygen causes a progressive fall in the size, but no change in the shape, of the heat response to stimulation. Fifteen hours of asphyxiation at 0° C. reduces the total response to one half, but recovery is relatively unaltered. This is further evidence for the existence, in nerve, of some form of oxidising reserve.

(12) Previous values of the ratio (initial heat):(total heat) are too high owing to the fact that a considerable amount of recovery heat was included in the estimated initial heat. For a short stimulus at 20° C. the initial heat is not more than 4 or 5 per cent. of the whole.

(13) The recovery heat occurs in two phases, one (A) of high initial rate, falling rapidly and ending in a few seconds, the other (B) of low initial rate, falling slowly and lasting probably for an hour. Regarding these as separate processes, the former (A) is of the same order of size as the initial heat and may

be directly connected with it ; the latter (B) is very much greater and possibly represents a "secretory" activity by which the ionic state of the nerve is restored after the electromotive changes of activity.

(14) Employing a double exponential curve to represent these two factors, it is possible to calculate the rate of heat production during and after a stimulus of any length or distribution ; the result has the same characteristics as the heat production actually observed.

(15) The possibility that the initial heat is due to phosphagen breakdown or lactic acid formation is discussed : the quantities available are sufficient. It may be that the initial process and the (A) process in recovery are analogous to similar processes in muscle. It is possible, however, that the initial heat is due simply to the discharge, or the partial discharge, of an electrical capacity distributed along a surface in the fibre.

(16) Some numerical data and analyses are given in Appendix I.

My thanks are due to Mr. A. C. Downing for constructing the thermopile which made this work possible ; to Dr. P. Auger for introducing me to the cuprous oxide photoelectric cell by which the galvanometer amplification was effected ; to Mr. W. Hartree for much advice and assistance with the analyses ; to Mrs. M. Beresina for her help with the experiments and in analysing many of the records ; to Messrs. T. P. Feng and S. L. Cowan for occasional willing aid ; and to Mr. J. L. Parkinson for his continual loyal assistance throughout the investigation.

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APPENDIX I.

NUMERICAL RESULTS.

Analyses, etc.

(A) *Experiment of December 2, 1931. 10^{-6} volt = 530 mm. Rapid galvanometer with amplification. 18.3° C. See fig. 11.*

Time (seconds) :	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3
$\frac{1}{2}$ second stimulation—							
Reading	0	0	7	41	94	150	197
Result	0.09	0.08	0.051	0.028	0.023	0.021	0.020
Remainder	0	-1	-4	0	2	2	-3
1 second stimulation—							
Reading	0	0	6	44	118	213	306
Result	0.09	0.15	0.095	0.073	0.05	0.038	0.033
Remainder	0	-1	-5	-4	-3	-1	1
2 seconds stimulation—							
Reading	0	1	13	54	131	258	415
Result	0.09	0.15	0.18	0.205	0.12	0.085	0.070
Remainder	0	0	2	3	-1	0	0
4 seconds stimulation—							
Reading	0	1	9	40	122	249	418
Result	0.08	0.15	0.18	0.205	0.215	0.215	0.223
Remainder	0	0	-1	-6	-4	-1	1
Control	0	8	109	354	639	847	955

Appendix I. (A)—(continued).

Time (seconds) :	3½	4	4½	5	5½	6	6½
$\frac{1}{2}$ second stimulation—							
Reading	234	262	284	300	316	325	331
Result	0.019	0.018	0.017	0.016	0.015	0.0145	0.014
Remainder	—2	—3	—2	—4	1	1	—2
1 second stimulation—							
Reading	383	437	480	508	531	547	561
Result	0.031	0.029	0.028	0.027	0.025	0.024	0.023
Remainder	1	—3	—2	—2	—3	—4	—4
2 seconds stimulation—							
Reading	563	703	802	875	930	975	1012
Result	0.065	0.060	0.056	0.053	0.051	0.048	0.045
Remainder	—6	—3	1	2	2	4	1
4 seconds stimulation—							
Reading	600	808	1023	1250	1442	1598	1712
Result	0.226	0.19	0.14	0.11	0.095	0.087	0.081
Remainder	—7	—5	—4	7	16	2	1
Control	993	992	970	939	906	873	841

Time (seconds) :	7	7½	8	8½	9	9½	10
$\frac{1}{2}$ second stimulation—							
Reading	337	343	348	352	355	—	—
Result	0.0135	—	—	—	—	—	—
Remainder	—2	—3	—1	0	4	—	—
1 second stimulation—							
Reading	574	583	592	598	605	609	613
Result	0.022	0.021	0.020	0.019	0.018	—	—
Remainder	—2	—2	1	1	3	4	3
2 seconds stimulation—							
Reading	1038	1058	1068	1078	1087	1094	1098
Result	0.042	0.039	0.036	0.033	0.030	—	—
Remainder	1	—1	—4	—3	—3	—3	—2
4 seconds stimulation—							
Reading	1800	1862	1913	1944	1972	1985	2000
Result	0.077	0.074	0.071	0.069	0.068	—	—
Remainder	—3	—7	2	—4	3	1	0
Control	810	781	754	728	704	680	657

The readings are the mean values, in units of 0.0268 mm., of 48 records ($\frac{1}{2}$ second), 36 records (1 second), 15 records (2 seconds), 12 records (4 seconds). The control was constructed from the instantaneous heating control (C) by adding C at 0 second to C at $\frac{1}{2}$ second and dividing by 2. Its value is 633 ergs = 15.1 microcal. per gram. The "results" of the analysis are given in fractions of this control.

Appendix I (B).

(B) *Experiment of January 8, 1932. 0° C. 16 seconds stimuli. Mean of 30 records in 1/30 mm. 10⁻⁶ volt = 211 mm. Rapid galvanometer with amplification. See fig. 14 (upper).*

Time (seconds) :	0	1	2	3	4	5	6	7	8	9	10
Deflection—											
Live	0	7	46	104	172	244	316	387	456	522	585
Control	0	64	487	778	839	794	733	679	631	587	548
Result	0.078	0.080	0.082	0.090	0.098	0.103	0.105	0.106	0.107	0.108	0.108
Remainder	—	2	3	—1	—1	1	2	2	2	—1	—1

Time (seconds) :	11	12	13	14	15	16	17	18	19	20	21
Deflection—											
Live	645	702	756	808	857	902	937	956	955	939	914
Control	513	482	454	429	406	385	364	344	326	310	295
Result	0.108	0.109	0.109	0.109	0.109	0.063	0.053	0.043	0.031	0.027	0.025
Remainder	0	—1	0	3	3	3	—1	—1	1	0	—1

Time (seconds) :	22	23	24	25	26	27	28	29	30	31	32
Deflection—											
Live	886	856	825	794	763	732	702	672	643	615	588
Control	281	268	256	244	233	223	213	204	195	188	181
Result	0.020	0.015	0.015	0.010	0.010	0.008	0.008	0.005	0.005	0.005	0.005
Remainder	1	1	—1	1	—2	2	—1	1	1	1	—

The "result" is in the terms of the control unit, which at the sensitivity given is 1820 ergs = 43.5 microcal. per gram.

(C) *Experiment of January 25, 1932, at 19.2° C. 16 seconds stimuli. 27 records, not all maximal stimuli. Results of analysis in microcal. per g. after multiplying by ratio (mean of greatest 4 records) : (mean of all 27 records) = 1.33 to give effect of maximal stimulus. Zc galvanometer, no amplification. See records in fig. 7 and results of analysis in fig. 2.*

Time (seconds) :	0	1	2	3	4	5	6
Σ 27	0	41	100	250	454	694	910
Heat leak correction	0	—1	—1	—2	—3	—4	—5
Σ 27 corrected	0	40	99	248	451	690	905
Σ 27/6*	0	7	17	41	75	113	151
Control, 1 second heating†	0	132	617	972	1081	1046	956
Result, C units	0.022	0.028	0.032	0.0355	0.0385	0.041	0.043
Remainder	0	4	0	—1	—1	3	2
Microcalories per gram	7.3	9.7	11.0	11.8	12.9	13.7	14.3

Appendix I (C)—(continued).

Time (seconds) :	7	8	9	10	11	12	13
Σ 27	1142	1353	1576	1775	1975	2160	2343
Heat leak correction	-5	-5	-6	-7	-7	-7	-7
Σ 27 corrected	1137	1348	1570	1768	1968	2153	2336
Σ 27/6*	189	225	260	294	327	360	390
Control, 1 second heating†	868	792	729	676	629	586	548
Result, C units	0.0445	0.0455	0.046	0.0465	0.047	0.0475	0.048
Remainder	2	0	0	0	0	0	0
Microcalories per gram ..	14.9	15.2	15.4	15.5	15.7	15.8	15.9

Time (seconds) :	14	15	16	17	18	19	20
Σ 27	2513	2686	2846	2974	3023	3007	2969
Heat leak correction	-6	-6	-5	-4	-2	-1	0
Σ 27 corrected	2507	2680	2841	2970	3021	3006	2969
Σ 27/6*	419	447	474	491	504	501	495
Control, 1 second heating†	514	483	454	428	404	383	364
Result, C units	0.0482	0.0483	0.029	0.023	0.021	0.020	0.018
Remainder	0	0	0	-5	-2	-2	0
Microcalories per gram	16.1	16.2	9.7	7.7	7.0	6.6	6.0

Time (seconds) :	21	22	23	24	25	26	27
Σ 27	2911	2837	2765	2695	2630	2573	2507
Heat leak correction	2	4	7	11	14	18	20
Σ 27 corrected	2913	2841	2772	2706	2644	2591	2527
Σ 27/6*	485	474	462	451	440	432	422
Control, 1 second heating†	346	329	313	298	284	271	258
Result, C units	0.015	0.014	0.014	0.014	0.014	0.014	0.014
Remainder	0	0	0	0	4	3	3
Microcalories per gram	5.1	4.5	4.5	4.5	4.5	4.5	4.5

* Smoothed by differences.

† Control = 251 microcal. per gram.

(D) *Experiment of February 9, 1932. 0° C. 16 seconds stimuli. 24 records, not all maximal stimuli. Results given in microcal. per g., after multiplying by ratio (mean of greatest 6 records) : (mean of all 24 records) = 56/52, to give effect of maximal stimulus. Zc galvanometer, no amplification. See fig. 19 for results of analysis.*

Time (seconds) :	0	1	2	3	4	5	6	7	8	9	10
Σ 24 in 0.2 mm.*	0	3	31	74	140	214	293	373	453	531	609
Control†	0	74	351	623	781	844	850	822	779	731	684
Result†	1.65	1.93	2.18	2.33	2.46	2.55	2.63	2.70	2.76	2.78	2.80

Appendix I (D)—(continued).

Time (seconds) :	11	12	13	14	15	16	17	18	19	20	21
Σ 24 in 0.2 mm.*	686	761	833	901	965	1023	1073	1115	1143	1153	1155
Control†	640	599	562	529	499	471	444	420	398	378	360
Result‡	2.84	2.86	2.89	2.89	2.89	2.13	1.85	1.61	1.48	1.36	1.25

Time (seconds) :	22	23	24	25	26	27	28	29	30	31	32
Σ 24 in 0.2 mm.*	1157	1152	1142	1124	1106	1088	1072	1056	1040	1024	1008
Control†	343	327	311	298	285	272	260	250	240	230	220
Result‡	1.18	1.10	1.06	1.06	1.05	1.00	1.0	1.0	1.0	1.0	1.0

* Corrected for heat leak. † Control = 556 microcal. per gram.

‡ Microcal. per gram for a single stimulus.

(E) *Tests of Analysis.* Various tests of the analysis were made of which the following, from the first and the last experiments performed, may be quoted.

(I) *Records for 6 seconds heating, analysed by $\frac{1}{2}$ second heating control.*

Time (seconds) :	0	$\frac{1}{2}$	1	$1\frac{1}{2}$	2	$2\frac{1}{2}$	3	$3\frac{1}{2}$	4	$4\frac{1}{2}$	5
Deflection—											
6 seconds heating	0	10	58	142	255	379	509	640	764	880	989
$\frac{1}{2}$ second heating	0	71	318	618	850	963	1000	990	956	917	875
Result, C units	0.15	0.13	0.12	0.125	0.14	0.14	0.115	0.13	0.12	0.13	0.125
Remainder	0	$-\frac{1}{2}$	-1	-1	$-\frac{1}{2}$	$-\frac{1}{2}$	$-\frac{1}{2}$	$\frac{1}{2}$	0	$-\frac{1}{2}$	0

Time (seconds) :	$5\frac{1}{2}$	6	$6\frac{1}{2}$	7	$7\frac{1}{2}$	8	$8\frac{1}{2}$	9	$9\frac{1}{2}$	10
Deflection—										
6 seconds heating	1093	1191	1282	1336	1347	1328	1288	1237	1187	1138
$\frac{1}{2}$ second heating	835	797	761	728	698	670	643	618	594	572
Result, C units	0.135	0.006	0	0	0	0	0	0	0	0
Remainder	0	$-1\frac{1}{2}$	$\frac{1}{2}$	0	$-1\frac{1}{2}$	$1\frac{1}{2}$	3	$\frac{1}{2}$	$2\frac{1}{2}$	$2\frac{1}{2}$

The deflection for 6 seconds heating is the mean of 2 records only (in 0.05 mm.). Nevertheless, and in spite of the fact that in this experiment the recording system was slow, the analysis with negligible remainders, gave a practically constant rate of heat production during the 6 seconds heating, and a drop to zero at the end.

(II) *Synthesis of 6 seconds heating control from 1 second heating control (mean of 4 records), for comparison with observed (mean of 3 records).*

Time (seconds) :	0	6	12	18	24	30
Observed	0	581	702	491	359	272
Synthesised	0	586	708	487	352	267

The average difference between the two is less than 1 per cent. of the maximum deflection, (800).

*A Method of Separating Antibodies from Serum Proteins.
Investigations on a Protein-free Antibody.*

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Working with diphtheria antitoxin and antityphoid serum, Frankel and Olitzki (1930) demonstrated that it is possible to separate the active antibodies from the serum proteins. The solutions of antibodies obtained were free from protein as indicated by sensitive chemical reactions and confirmed biologically by sensitisation tests. It was found (Olitzki and Frankel, 1931) impossible to sensitise guinea pigs by injecting such solutions of antibodies both against the solutions themselves as well as against the original untreated sera.

The present paper contains (1) a complete description of a method of separating antibodies from serum proteins (including improvements made subsequent to previous publications), (2) the results of purification and concentration of antibody and chemical investigations on the protein-free flagellar typhoid agglutinin, particularly the action of proteolytic enzymes.

A Method of Separating Antibodies from Serum Proteins.

In principle the method consists of adsorption of the antibodies and of other constituents of the original serum on kaolin followed by elution of the adsorbate by aqueous solutions of certain organic compounds which liberate the antibodies only from the kaolin. Both adsorption and elution depend largely on the physico-chemical, and particularly the colloidal, properties of the serum and the kaolin employed. The qualities of different samples of kaolin as well as the origin, the manufacture and method of preserving sera appear to influence the results. It is therefore necessary to modify proportions, time and temperature according to the materials employed, but these modifications do not affect the main principle.

The actual details given in previous publications were valid only for the serum and kaolin then used, and in order to fix suitable conditions for new material the following experiments were carried out.

Determination of the Optimum Conditions for Adsorption.

Gradually increasing quantities of kaolin are added to a series of centrifuge tubes containing equal amounts of serum (diluted 1 to 9 normal saline). The kaolin is added gradually and the mixtures stirred in order to prevent the formation of clumps. Amounts of kaolin varying between 1 to 5 gm. are added to each tube containing 10 c.c. of diluted serum. The results obtained serve as indications for further experiments. The series of tubes are tightly corked and placed in a shaking apparatus working at a uniform rate which can be reproduced accurately. At certain intervals (*e.g.*, 1 hour in the first part of the adsorption and subsequently every 4 or 5 hours) samples are withdrawn from each tube and centrifuged. The supernatant fluid is drawn off and tested for proteins and antibodies. (If it is opaque it should be filtered.) When a sample is found which is free from antibody, the experiment is stopped. In this way the conditions for obtaining complete adsorption of antibodies in the shortest possible time and with a minimum amount of kaolin is found.

In some cases the antibodies and the proteins are completely fixed simultaneously, in other cases quantities of kaolin which are insufficient to adsorb all the proteins effect the complete fixation of the antibodies. It is necessary to determine these points with every sample of serum and every sample of kaolin and the supernatant fluid is therefore examined for both proteins and antibodies.

As previously stated, the quantities of kaolin and the time required for the complete adsorption of antibody from a given serum vary with different samples of serum and kaolin and once the optimum conditions of adsorption have been determined no factor should be changed.

In previous communications dealing with this subject (Frankel and Olitzki, 1930; Olitzki and Frankel, 1931) we had to use equal parts of 1 in 10 diluted serum and kaolin and to allow the suspension to stand about 24 hours in order to effect a complete adsorption. By carrying out the operation with continuous shaking as described above and by using other samples of kaolin (from the same manufacturer) it was found that 20 to 40 per cent. of kaolin was sufficient to cause complete adsorption within 3 hours.

In order to get an adsorbent of uniform properties from different samples of commercial kaolin we tried to submit them to various treatments, but without satisfactory results. In the present state of knowledge of the relation between physico-chemical properties and adsorption it is necessary to test every sample individually.

The Optimum Conditions for Effecting the Specific Extraction (Elution) of the Antibodies from the Adsorbate.

Under certain conditions substances such as glycerol, saccharose, glyocol, etc., in aqueous solution become effective eluents; glyocol in particular gave satisfactory results. This amino acid was first used by Fodor and co-workers (1925) to elute peptide splitting enzymes which were obtained practically free from protein.

In the experiments referred to in the present paper mixed solutions of 2 per cent. of glyocol and different amounts of sodium chloride were found to be better eluents than pure solutions of glyocol. In many cases only the addition of a certain amount of the inorganic salt converted an inactive solution into an effective eluent.

With the glyocol-sodium chloride as eluent there are three important factors which influence the results:—

- (1) The relation of the quantity of the adsorbate to the volume of the eluting solution.
- (2) The temperature at which the process is allowed to take place.
- (3) The amount of sodium chloride added to the solution containing 2 per cent. glyocol.

(1) As a rule a volume of eluting solution equal to the original volume of diluted serum was used; sometimes a greater quantity of the glyocol-sodium chloride solution was advantageous.

(2) The elution was carried out (at least partially) at 37°. In case it should not be possible to shake continuously at this temperature the shaking may be interrupted and the tubes allowed to stand for some time, e.g., overnight at 37°.

(3) As mentioned above, the concentration of glyocol was fixed at 2 per cent. while that of sodium chloride was varied; the optimum relation has to be determined by experiment.

It seems that the specific efficiency of the combination of the amino acid and the inorganic salt is due to the formation of addition compounds in aqueous solution (Pfeiffer, 1927); these new compounds have different chemical properties from their components and they are more specific eluents, e.g., it was found that a solution containing 2 per cent. of glyocol and 2 per cent. of sodium chloride extracts only the flagellar agglutinin from an adsorbate containing both the flagellar and the somatic typhoid agglutinins, while an eluting fluid

consisting of a 2 per cent. solution of glyocol and only 0.3 to 0.5 per cent. sodium chloride liberates mainly the somatic agglutinin.

On the principles indicated above it is possible to determine the optimum conditions for effecting elution in every case by carrying out suitably arranged sets of experiments.

The suspension containing the adsorbate, *e.g.*, the kaolin charged with the antibodies and other components of the original serum is centrifuged, the supernatant fluid removed, the residue resuspended in saline or water, shaken for a short time and then again centrifuged and washed. The supernatant fluids are tested for antibodies and for proteins, because some protein and occasionally a trace of antibodies are removed by washing. If this preliminary step is omitted those proteins which are only lightly bound to the kaolin may appear in the final elution.

The adsorbate is weighed, divided into equal parts—according to the number of intended parallel experiments—which are then distributed in centrifuge tubes and to each tube a solution of eluent is added. From time to time samples are drawn off and tested for antibodies and proteins. The experiments are broken off when the continued elution does not increase the content of the antibodies in the solution. With different sera and samples of kaolin this point was reached in 20 to 70 hours.

The tests for proteins should be carried out under the most rigorous conditions in order to ensure that no traces of protein remain in the solution containing the antibodies. For this reason precipitation tests are always allowed to stand for 20 hours before reading.

As a rule the solution of antibodies obtained by elution does not contain any protein. Should a trace of protein be found, it is possible to remove it by a second careful adsorption with kaolin. In principle only the smallest amounts of kaolin should be used and the shaking carried out in the shortest possible time. By using too large a quantity of the adsorbent the solution loses a large part or all the antibodies together with the traces of proteins; from these second adsorbates the author never succeeded in recovering antibodies by further elution.

Sometimes it was advantageous to re-adsorb the traces of proteins fractionally from the eluates, that is, to add gradually the small quantity of kaolin in parts and to remove the kaolin charged with the proteins before every further addition of adsorbent.

In order to recover as large a part as possible of the antibodies primarily adsorbed on kaolin it is advisable to repeat the elution several times, taking care to replace the eluting fluid by new solution after each extraction.

As in the case of enzymes, some antibody is always lost in the processes of adsorption and elution. It is possible to remove the greater part of the serum proteins without any considerable diminution of serological activity, but the separation of the antibodies from the last traces of protein involves heavier losses, varying with serum, kaolin and details of the operations.

In a large number of experiments carried out with different curative sera protein-free antibodies were obtained by the above method.

For example, with (I) *Diphtheria antitoxin*.—The original serum was labelled 1000 I.U. per cubic centimetre. The antitoxin content of both the original serum and the protein-free eluates was determined by the intracutaneous neutralisation test employed by Roemer (1909). The determinations were carried out by L. Olitzki. The eluting fluid consisted of a solution containing 2 per cent. glyocol and 1 to 2 per cent. sodium chloride.

Table I.

Experiment.	Titre of original serum per cubic centimetre (skin units).	Titre of the eluate calculated per cubic centimetre of original serum.	Yield of recovered antibody (antitoxin).
			Per cent.
1	5000	1000	20
2	5000	1200	24
3	5000	600	12

Note.—For the protein tests each 10 c.c. of eluate (obtained from 10 c.c. diluted original serum = 1 c.c. original serum) were concentrated to 1 c.c. under low pressure at a temperature not exceeding 30°. For particulars of tests and for a protein test with the solid residue of evaporated eluate see under tests for proteins.

(II) *Antityphoid Serum*.—The agglutinin content of both original antityphoid serum and protein-free eluates was measured by the usual agglutination test.

Table II.

Experiment.	Titre of original serum per cubic centimetre.	Titre of eluate calculated per cubic centimetre of original serum.	Yield of recovered antibody.
			Per cent.
1	5000	1200	24
2	5000	1000	20
3	5000	1100	22
4	40000	3000* 1100† 900‡	12·5

* First elution.

† Second elution.

‡ Third elution.

Note.—Eluates for the protein tests were concentrated as in the case of antitoxin.

Tests for Proteins.—Both precipitation and colour tests were tested with regard to their sensitiveness and it was found that under the conditions of this investigation the former gave the more satisfactory results. The Esbach and the Spiegler tests were most suitable. With both of them it was possible to detect the presence of proteins in a solution containing 0·002 per cent. serum proteins. To the filtered eluates a few drops of reagent and in the case of the Esbach test some acetic acid were added. Should the solution remain clear it is gently warmed and allowed to stand at least overnight. Traces of proteins may be detected as a light precipitate at the bottom of the test tube. Millions' reagent is not so sensitive as the Esbach or Spiegler test, carried out in the manner described. In addition to the precipitation tests it was used when no phenols were present. For dialysed, glycol-free elutions the Ninhydrin test was used as well. With pure eluates all these protein tests gave negative results.

In the course of the continued investigation on the flagellar agglutinin there were opportunities of determining whether more concentrated solutions of the antibody (with a titre of 10,000) contain any protein, but none was found.

In order to carry out the search for proteins under as favourable conditions as possible for their detection glycol-free eluates of antibody were evaporated to dryness, the solid residues dissolved in a few drops of a solution of Ninhydrin and heated for some time in boiling water. Ninhydrin was chosen as reagent because it appears that this sensitive reaction is common to all proteins and to their decomposition products. This test also gave negative results.

The active eluates may therefore be considered as protein-free.

For sensitisation tests see Olitzki and Frankel (1931).

Evidence for the non-protein nature of the antibody is also furnished by the action of pancreatin on the eluate (see the last section of this paper).

It may be mentioned here that it was possible to obtain protein-free, serologically active ultrafiltrates of antityphoid sera (Serotherapeutical Institute of the State, Vienna) by carrying out ultrafiltrations with Bechhold filters (containing 3 per cent. glacial acetic acid-collodium) under pressure. Such filtrates contained only small amounts of the flagellar agglutinin (3 to 6 per cent. of the original titre).

Further Concentration and Purification of Eluates.

The experiments referred to below were carried out with eluates containing mainly the protein-free flagellar typhoid agglutinin; this antibody was chosen

for the first chemical investigations because of the ease with which it can be estimated by agglutination tests.

Further purification was attempted in order to determine—

- (a) Whether the specific eluent is essential to the activity of the antibody ;
and
- (b) Whether the antibody is a colloid or a crystalloid.

Dialysis of the protein-free eluates containing the agglutinin was carried out. The membranes used consisted of small parchment-sacks (Schleicher and Schuell) which were previously examined for tightness and permeability and were allowed to soak for 24 hours in distilled water with the addition of thymol. Sterile water was used as external fluid and was renewed every 3 or 4 hours. The parchment-sacks containing the antibody in glyccol-sodium chloride solution were placed in the water and the external and internal fluids were kept at the same level in order to avoid changes in the volume of the solution of antibodies. The dialysis was carried out under practically sterile conditions without the addition of preserving material. The glyccol disappeared from the internal solution after some hours of continued dialysis, but at least 48 hours were required before the chloride reactions became negative. The solutions contained in the parchment-sacks were then pipetted off. Changes in the volume of the solution which occurred during the dialysis must be observed in order to be taken into account in determining the agglutinating titre.

The dialysed solution even when concentrated does not show any glyccol and practically no chloride, but contains the whole or at least almost the whole amount of antibody present in the eluate previous to dialysis. It is therefore evident that the eluted flagellar typhoid agglutinin does not require the presence of glyccol-sodium chloride for serological activity and that the protein-free antibody has colloidal qualities.

The next step in concentrating the flagellar agglutinin is to convert it into the solid state.

The dialysed solution is evaporated to dryness under a pressure of not more than 3 or 4 mm. at room temperature or a little above room temperature.

Agglutination experiments carried out with the dried and redissolved agglutinin showed that practically no loss of serological activity occurs during drying, if the conditions mentioned above are observed.

The solid residue containing the whole active principle of the dialysed solution is remarkably small and could be weighed only on a microbalance. The

Table III.

Protein-free eluate of flagellar agglutinin not dialysed.	Protein-free eluate after dialysing.	Redissolved residue of dialysed eluate.
Titre. 3000 1000 720	Titre. 2500 900 720	Titre. 2500 900 650

weight varies with different sera. The residue contains the antibody in a very concentrated form, but it is not suggested that it contains only antibody.

Search for Lipoids in the Residue.

The solid residue was dried in a desiccator over concentrated sulphuric acid under low pressure till constant weight was reached and then repeatedly extracted with pure, absolute ether. The collected ether-extracts were filtered and then evaporated to dryness at room temperature. Only smallest traces of an oil remained which were imponderable. Practically all of the residue was insoluble in ether and remained unchanged after extraction with ether.

Comparative determinations of the flagellar typhoid agglutinin were arranged with solutions of the following substances :—

- (1) The original solid residue (not treated with ether) as obtained by evaporating and drying the dialysed elution of the antibody.
- (2) The ether-soluble traces of the original solid residue.
- (3) The ether-insoluble fraction of the original solid residue.
- (4) Finally with a solution containing both the ether-insoluble fraction and the ether-soluble traces of the original solid residue.

Table IV.

Solution	(1)	(2)	(3)	(4)
Titre	1000	0	900	900

The results of these agglutination tests showed that there is practically no difference in the activity of the original solid residue before and after extraction with ether. The ether-soluble traces do not give any agglutination, while the same titre was found in the recombination of the ether-insoluble and the ether-soluble fraction as in the ether-insoluble fraction itself.

It is therefore evident that the solid, protein-free preparation of the typhoid agglutinin contains practically no lipid and that the removal of the traces of ether-soluble substances does not alter the serological activity of the remaining antibody.

Effect of Proteolytic Enzymes on an Eluate containing Flagellar Agglutinin Free from Protein and on the Original Antityphoid Serum.

It has been shown both by chemical reactions and the more susceptible biological sensitisation test that solutions of antibodies obtained from the original antisera by the method described above do not contain proteins. As a further test for proteins in such solutions the action of a natural system of proteolytic enzymes on eluates was studied.

If the eluate were to contain *traces of protein* which were not detected either by chemical reactions or by the biological sensitisation test but were of *importance for the biological activity* of the antibody, it would be expected that the hydrolysis of these traces of protein by the action of enzymes would be followed by the destruction or at least by considerable losses of the antibody.

At the same time the effect of hydrolysis of the proteins of an antityphoid serum on the agglutinin was studied. For these experiments a highly concentrated antityphoid serum was used which, according to the manufacturer (Serotherapeutical Institute of the State, Vienna), was free from lipoids and contained only those fractions of the serum proteins from which it was not possible by the methods used till now to separate the agglutinins and which were believed to be inseparably bound to the antibodies.

For our experiments the enzyme preparation Pancreatin (Parke, Davis & Co.) was used which contains proteolytic enzymes of high hydrolysing activity in alkaline medium, $p_H = 8$. It was ascertained that such a grade of alkalinity does not affect the agglutinin. The hydrolysis of the proteins was measured by usual titration according to Sørensen.

All experiments with the enzymes were carried out under optimum conditions for the hydrolysis of proteins at a temperature of 37° and extended over 80 hours.

The active, protein-splitting enzymes had no influence at all on the agglutinin contained in the eluates. This fact is a further proof for the non-protein nature of the serologically active principle and confirms both the chemical and the biological sensitisation tests.

In the case of the concentrated original antityphoid serum containing only those fractions of the serum proteins believed to be either inseparably connected

with the antibodies or identical with them, the pancreatin effected a strong hydrolysis of the proteins, as measured by the very considerable increase of free amino groups. In this case also no loss of agglutinin could be found, for the titre remained quite unchanged. It is therefore evident that those proteins are not essential for the activity.

Summary.

A method is fully described by which antibodies can be separated from serum proteins and lipoids by adsorption and elution.

Investigations were carried out with the protein-free flagellar typhoid agglutinin.

The antibody can be freed from the eluent by dialysis without loss of serological activity.

The protein-free antibody shows colloidal properties.

It was obtained in solid form ; there was no loss of serological activity after redissolving in saline.

The removal of ether-soluble traces from the solid residue has no influence on serological activity.

Proteolytic enzymes have no effect on the eluted antibody ; they hydrolyse the proteins of antityphoid serum without affecting the agglutination titre.

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Central Nerve Terminals in the Mammalian Spinal Cord and their Examination by Experimental Degeneration.

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[PLATES 8 AND 9.]

I. *Introduction.*

Since the discovery by Held (1897, 1902) of the *boutons terminaux* in the central nervous system, and the confirmation of this discovery by Auerbach (1899) and Cajal (1908), the function of these structures as synapses has gradually come to be recognised. The present study is an attempt to re-investigate these boutons and their relations to nerve cells in the cat; and also to observe the changes they undergo following section of the nerve fibres of which they are the terminals.

II. *Technique.*

The silver methods of staining the nervous system have been often criticised in that they did not give consistent results. It is believed that this is due, partly at least, to imperfect fixation. Nervous terminals of the grey matter are highly susceptible to post-mortem destruction, and with the ordinary method of placing small pieces of tissue into a quantity of fixative, it is quite likely that changes occur in the finest fibres and their terminals before they are fixed. Further, it has been found that the presence of blood in the capillaries of the grey matter tends to make the tissue refractory to the silver stain. In these studies, therefore, the method of injecting the fixative into the living animal has been used, with the result that in successful impregnations all of the intra-central nerve terminals have been stained.

This injection procedure is simple and easily carried out: the cat is anaesthetised with pure ether, and a tracheal cannula inserted so that artificial respiration may be used if necessary. The abdomen of the supine animal is opened by a medial longitudinal incision, and the viscera pushed to one side; next, the two renal arteries, the inferior and superior mesenteric arteries, and the coeliac axis are clipped with hæmostatic forceps. A cannula is inserted into the abdominal aorta at the point of its bifurcation, and a 10 per cent. solution of chloral hydrate in distilled water is injected towards the heart. At the same time, the inferior vena cava is opened and injection continued until clear

fixative flows from it. In practice, a cat of 2 kilograms requires about 150 c.c. of solution. The central nervous system is then removed immediately, cut into small pieces about 1 cm. thick, suitably labelled, and put into a large excess of 10 per cent. chloral hydrate for 24 hours. The pieces are next treated according to Cajal's formula (6A) (Bolles Lee, 1928). They are kept in 96 per cent. alcohol with 7 drops of ammonia per 100 c.c. for 24 hours; they are then blotted dry and incubated for 6 days at 37° C. in 1.5 per cent. silver nitrate in distilled water. It was found beneficial to change the silver nitrate after 3 days; also, care must be taken to use chemically clean glassware, pure distilled water, and to see that no metal comes into contact with any of the solutions.

Before being reduced in 2 per cent. aqueous solution of hydroquinone, the pieces are washed in running distilled water. The time of washing is important, and it was found that with pieces of 1 cm. thickness one half-hour of washing was sufficient. Reduction is complete in 24 to 48 hours; the pieces are then dehydrated, embedded in paraffin wax, and sectioned at 15 μ .

Successful preparations have a purple tinge; the nerve cells are red to purple with the intracellular neurofibrils darkly stained and the nuclei standing out sharply in a lighter shade. The nucleolus is yellow to brown. In the white matter, the axis cylinders are purple to black, while the medullary sheath is dissolved away leaving an unstained space around each axis cylinder.

III. *The Normal Synapse of the Cat.*

A. *Development.*—In preparations of new-born kittens it is impossible to observe any boutons on any of the cells in the cord. In a series of such animals ranging in age from 1 day to 2 months, the first boutons were seen the twenty-first day after birth. This confirms the observation of Windle (1930), who saw boutons in 12 to 21-day old kittens. In the author's preparations, the earliest type observed were the *boutons de passage*.

B. *The Normal Boutons of the Adult Cat.*—The *boutons terminaux* are small, oblong loops occurring at the end of nerve fibres and applied to cell-bodies and their dendrites. The *boutons de passage* are similar structures which are found along the length of the finest nerve fibres, on both cell- and dendrite-surfaces. Boutons have been seen on the cells of the spinal cord, and medulla oblongata, on the dendrites of Purkinje cells, and the bodies of the nerve cells in the granular layer of the cerebellar cortex. They have been demonstrated also on cells of the cerebral cortex. The present studies, however, are concerned only with boutons of the spinal cord. On the ventral horn cells they

vary in dimensions from $2.2\ \mu$ by $1.1\ \mu$ to $1\ \mu$ by $0.5\ \mu$. The *boutons de passage* here are of similar width but are usually greater in length, some being $2.5\ \mu$ to $3\ \mu$ long. On funicular cells, the terminal boutons are normally $3\ \mu$ by $2\ \mu$ to $1.7\ \mu$ by $1.1\ \mu$, *i.e.*, they are on the whole slightly larger than ventral horn boutons. Moreover, very tiny boutons, less than 1 micron in length, and difficult to measure, may be found in any region of the cord and especially around ventral horn cells.

The boutons are distributed on the cell-bodies and dendrites without distinction as to size or whether they be terminal or passage types; there is, thus, as great a variation in size of boutons on the dendrite as on the cell-body surface, and the percentage of *boutons de passage* is the same on both. Hence, as far as the synapses are concerned, there is no structural basis for believing that dendritic processes differ in function from the cell bodies. Further, boutons occur not only at the proximal part of the dendrite as Tiegs (1931) believed, but also at great distances from the cell. On the cell-body, they are very dense; in an average example, illustrated in fig. 1, there were 100 boutons on an area $20.5\ \mu$ by $62.7\ \mu$ on the surface of a cell-body; in other

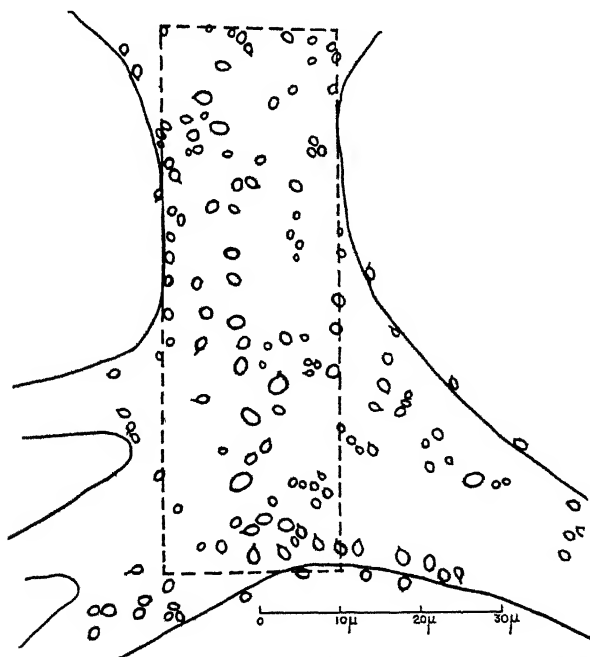


FIG. 1.—A normal nerve cell with the *boutons terminaux* and *boutons de passage* represented in outline. All of the boutons on one surface of the cell are shown. The area enclosed by the broken lines is $20.5\ \mu$ by $62.7\ \mu$ and contains 100 boutons. Cat 21. Fifth cervical segment. Magnification, 1092 diameters. (Camera lucida drawing.)

words, one bouton per $12.8 \mu^2$ of cell-surface. On some cells, they are as dense as one bouton per $9 \mu^2$, and on others as sparse as one per $17 \mu^2$. On the dendrites, they are less dense, and there is roughly one bouton to every $20 \mu^2$ of surface. The total number of boutons on a cell varies, of course, according to its size; the ordinary cells of the ventral horn have from 300 to 350 boutons on the cell-body surface alone. The cells of other regions of the grey matter, being smaller, have fewer terminals; for instance, there are about 100 boutons on the cell illustrated in fig. 14, Plate 9.

The projections of grey matter which extend into the ventral columns of white matter are also supplied with boutons. Here they are applied to thick fibres which are dendrites of cells located in the grey matter proper. Fig. 2

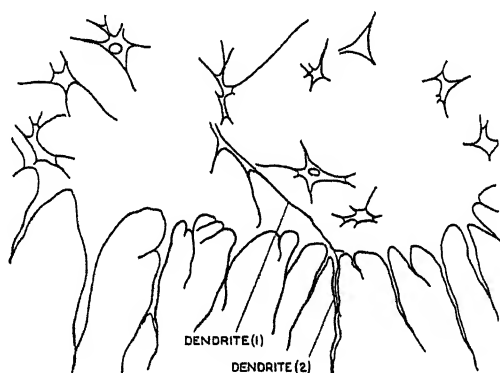


Fig. 2.—Part of the left ventral horn in the fifth post-thoracic segment of a cat's cord. A dendrite is shown proceeding from the grey matter proper into one of the projections of grey into white matter. Cat 24. (Camera lucida drawing.)

illustrates an example of such a cell-body which receives a dendrite running in from one of these projections. In fig. 3, which is a detailed drawing of part of the same outlying projection, two boutons are seen terminating on the dendrite. These are the terminals either of small nerve fibres passing out into the projection along with the dendrite, or of collaterals which enter from the white matter directly. Many examples of collaterals coming from axis cylinders of the white matter into the projections of the grey have been seen.

Surrounding the cell-bodies and dendrites are tiny fibrils which stain darkly, and run along the cell- and dendrite-surfaces. Around many cells, especially those of the ventral horn, they are so numerous as to constitute a nervous meshwork surrounding the cells and their processes. Careful analysis of this meshwork reveals neither branching nor anastomosis. The fibres pursue a

very tortuous course, twining themselves around the dendrites and either terminating by means of boutons or else passing out of the plane of section. A fibre may end in either of two ways: it may approach the cell gradually to end in its bouton; or it may pursue a course parallel to the cell-surface, turn abruptly at right angles and proceed to its termination.

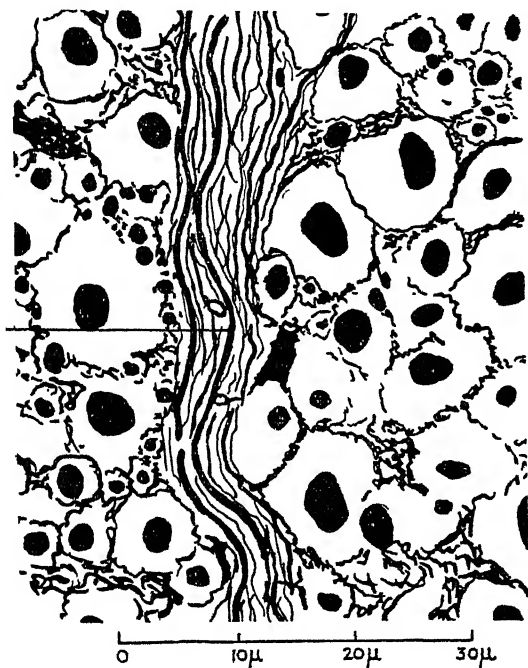


FIG. 3.—A detail of the grey matter projection in the region indicated in fig. 2 (dendrite (2)). Two boutons are seen to terminate upon the dendrite. Cat 24. Magnification, 1616 diameters. (Camera lucida drawing.)

It has not been possible to demonstrate continuity between the boutons and the intracellular neurofibrils. As can be seen in fig. 11, Plate 8, the boutons end freely on the cell-surface; often they are not orientated in the direction of the intracellular neurofibrils at all. In fig. 10, Plate 8, which shows a cell cut through and a bouton applied to its surface, there is no evidence of any connection whatever running from the bouton into the cell. Only one apparent case of fibrils proceeding from terminal boutons has been observed, fig. 8, Plate 8. On carefully examining this bouton, it is seen that the small forked process coming from the loop does not enter the cell, and certainly has no connection with the intracellular neurofibrils. It is to be concluded that this is an abnormal type of terminal of very rare occurrence.

IV. *Experimental Degeneration of the Boutons Terminaux.*

A. *Experiments on the Lumbar Enlargement.*—With a view to determining whether *boutons terminaux* could be modified experimentally, a series of de-afferented adult cats was aseptically prepared. With the cat under ether the afferent roots of the right fifth, sixth, seventh and eighth post-thoracic nerves were severed central to the ganglia, outside the theca. The animals recovered, and were killed 24 hours, 29 hours, 43 hours, 48 hours, 3 days, 6 days, 9 days, 13 days and 44 days after the operations. The cords were fixed by the injection method previously described, stained according to Cajal, and serial sections of the lumbar enlargement prepared.

Examinations of these sections revealed that in a clearly defined region in the cord just dorsal and lateral to the central commissure, the area indicated by large dots in fig. 4A, the boutons showed pronounced changes. These

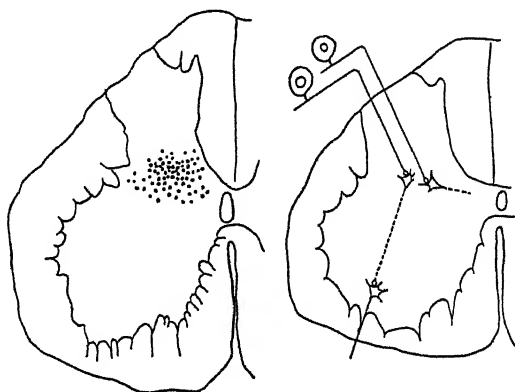


FIG. 4A.

FIG. 4B.

FIG. 4A.—An outline of the right half of the spinal cord at the seventh post-thoracic segment, from a cat completely de-afferented on the right side of the lumbar enlargement. The drawing was made from six sections, and each dot represents a cell upon which degenerating boutons were found. Cat 2, operation 2. (Camera lucida drawing.) FIG. 4B.—A diagram showing the manner of termination of afferent nerves in the grey matter of the cord suggested by the location of degenerated boutons in the grey matter of de-afferented cords. The way in which cells in the mid-region connect with ventral horn cells is not suggested, as implied by the broken line.

modifications were restricted to boutons of this region on the operated side, and terminals of all other cells were normal. In the cats killed 24 hours after the operation, there was a slight but noticeable enlargement of the boutons due to a swelling of the loop, which became thick, slightly granular, and stained darkly. The most typical appearance of this stage was the small fibril that

runs up the centre of the bouton. This structure is seen sometimes in boutons of normal cords, but not to the extent that it occurs in operated cats. This central filament often showed a little moniliform swelling, fig. 10, Plate 8, and in some boutons at this stage, the space on either side had become somewhat opaque. In the 29-hour cat, there were some boutons showing the same characteristics as those of later stages, and, as fig. 12, Plate 9, illustrates, some terminals on cells showing degenerating boutons remained normal.

Forty-eight hours after the operation, the boutons have become, for the most part, darkly staining; some are entirely opaque, while others are lighter in the centre than at the edges and show the tiny fibril observed at the 24-hour stage. There is a more pronounced irregularity and granulation of the boutons than previously, and considerable variety in shape. Some are round or pear-shaped, while others become greatly elongated. Accompanying these changes in shape and structure is a definite increase in size. This may be seen in fig. 5 which represents a cell from the cord of a cat killed 43 hours after

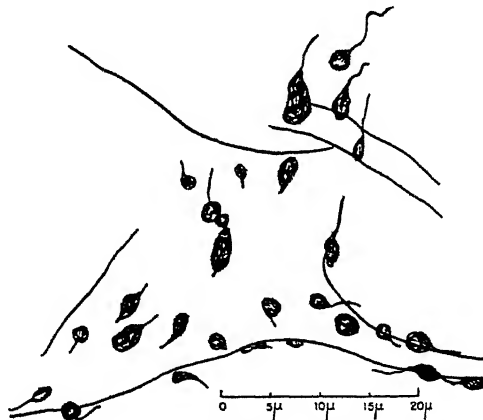


FIG. 5.—*Boutons terminaux* from a cord which had been de-afferented 43 hours previous to sacrifice of the cat. Cat 5, operation 6. Magnification, 1092 diameters. (Camera lucida drawing.)

de-afferentation. On this cell, the largest terminal was 4.6μ by 3μ ; others were of the order of 4.5μ by 1.5μ , and no degenerating terminals were smaller than 2.7μ by 1.5μ . The cell shown in fig. 14, Plate 9, is from the same section, but the boutons here are smaller than the average degenerating terminals of this stage, the largest ones being 3.5μ by 2.8μ , and most of them being about 3μ by 1.5μ .

After 3 days, the boutons are very swollen, elongated, and granular, while the loop can scarcely be distinguished. Their shape is variable, but they

are mostly long and spatulate, or pear-shaped and thick. Fig. 6 illustrates a cell in the cord of a cat killed 3 days after the operation, showing some of these variations. In this cell, bouton No. 1 is $5.3\ \mu$ by $3\ \mu$, No. 3 is $5.6\ \mu$ by $2.3\ \mu$, and No. 6 is $6.1\ \mu$ by $2.3\ \mu$, while others are about $4.5\ \mu$ by $1.5\ \mu$. Fig. 15, Plate 9, is a photograph of the same cell, and fig. 16, Plate 9, is an enlargement showing some of its boutons. On one terminal in this photograph, the remains of the central fibril can still be seen, and, as in all stages, some normal boutons are present.

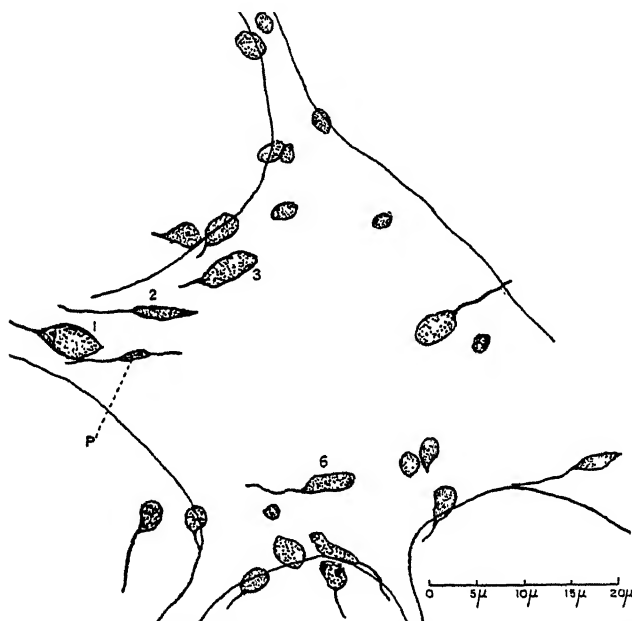


FIG. 6.—A cell from the operated side of the cord in the sixth post-thoracic segment. The animal was killed 3 days after de-afferentation. Boutons from this cell are shown in fig. 16, Plate 9. Cat 2, operation 2. Magnification, 1092 diameters. (Camera lucida drawing.)

After 4 days, many boutons are merely granular masses, and in the 6-day cats, terminals of abnormal type cannot be seen. In these cords, the operated side shows a scarcity of boutons in the degenerate area, those which remain being the terminals of fibres not involved in the operation.

As indicated in fig. 6 (P), the *boutons de passage* degenerate as well as the terminal boutons, following the same stages. The elongation and granulation are especially noticeable in them.

During all the stages described, the cell bodies and dendrites of the abnormal area, although covered with degenerate boutons, were normal as far as could

be ascertained by the silver method. The cells were unshrunk, the nuclei normal in shape and central in position, and the neurofibrils stained as in normal cords. It is concluded from this observation that there is no direct continuity between *boutons terminaux* and the intracellular neurofibrils. At least, the degenerative process ceases abruptly at the bouton and does not continue into the cell; one would not expect this result were there unbroken continuity.

B. *Experiments on the Cervical Enlargement.*—Another series of cats, de-afferented in the cervical enlargement, was prepared. Results similar to those in the lumbar enlargement were obtained. Degenerated boutons became well marked in the mid-region of the grey matter on the operated side, and as was the case in the lumbar enlargement, they occurred in this region only; abnormal terminals were not found on ventral horn cells of the same side nor on any cells of the opposite side. The times of degeneration and the appearance of the terminals were the same as in the lumbar enlargement.

C. *Longitudinal and Transverse Sections of the Cord.*—In adult cats, longitudinal medial incisions were made in the lumbar region from the third to the sixth post-thoracic segments. These incisions completely severed the dorsal and ventral commissures, and in some cases cut into the grey matter of the right or left side. In other cats, transverse semisections were made in the lumbar region. In these cords, degenerating boutons were also seen. For instance, a cord was medially incised between the third and sixth post-thoracic segments, the animal being killed 48 hours after the operation. Sections taken from the site of the lesions show cells upon which there are elongated, enlarged and granular boutons, which appear similar to those seen in cords de-afferented 3 days previously. In this case, degeneration was not restricted to any part of the grey matter, but was found on many cells near the lesions. In the semisected cords, sections taken above and below the lesions also showed some degenerate boutons. Here, again, they are found to be very widely scattered in the grey matter. It is believed that these experiments confirm the view that the changes in appearance of the boutons are actually degenerations due to cutting their nerve-supply.

D. *Section of the Motor Roots.*—The degeneration of boutons following de-afferentation was found to be restricted to cells in the mid-region of the grey matter. This leads to the conclusion that afferent nerves do not end directly upon ventral horn cells. Neither do they cross to the opposite side. It is concluded that afferent fibres terminate by means of boutons upon the cells of the mid-region, and that axones proceed from these to form synapses

with other cells. The simplest type of reflex in the cord, therefore, involves at least two intra-central synapses and one internuncial neurone.

Some cells of the region of degeneration might, however, send axones directly to the motor root. In order to determine whether or not this were so, efferent or ventral roots of the cord were cut in cats, under the usual aseptic conditions, and Cajal preparations made after fixation by vital injection. Six days after the operation it was possible to identify the cells whose axones were severed. The nucleus of such a cell is eccentric in position, the protoplasm surrounding the nucleus fails to impregnate, and the neurofibrils are not stained. Cells showing these changes were confined to the ventral horn, and in no instances were such degenerations found in cells of the mid-region. It seems reasonable to conclude, therefore, that the axones of cells in this mid-region do not leave the cord, and that they are purely associative in function.

V. Discussion.

It is concluded from these studies that the existence of boutons as actual structures cannot be denied. Although Tiegs (1926) was unable to demonstrate them in new-born and foetal animals, and therefore supposed they did not exist, it is now known that boutons do not occur in new-born animals such as rabbits and kittens, which are relatively undeveloped at birth. Tiegs maintained the view that "the fine collaterals that pass from the white matter into the grey matter, enter the dendrites either singly or in bundles, so that integration will occur within the body of the nerve cell, and not on a synaptic membrane which is applied to the nerve cell." He pointed out that defective staining methods resulted in vacuolation or frothing of the protoplasm at the point where a dendrite enters the cell. This gives an effect which he believed might be mistaken for a ramifying synapse.

The cells of frogs stained with methylene blue, which Tiegs illustrates (1926, opp. p. 76), undoubtedly show this artificial effect, as true boutons have not been stained by this method. With careful silver staining, however, actual boutons can be demonstrated on the spinal cord cells of adult frogs (unpublished observations of Hertz in this laboratory). Later (1931), Tiegs admitted the existence of boutons in adult animals.

Ballantyne (1925) studied the large Mauthner cells of *Lepidosiren paradoxus* and concluded that there was direct continuity between the extracellular nerve fibre and the intracellular neurofibril. She believed that the end-loop or bouton was an optical illusion due to the use of the monocular microscope.



Fig. 7. 0 10 μ 20 μ 30 μ



Fig. 9. 0 10 μ 20 μ



Fig. 10. 0 10 μ 20 μ



Fig. 8. 0 10 μ 20 μ



Fig. 11. 0 10 μ 20 μ 30 μ



Fig. 12. 0 10 μ 20 μ



Fig. 13. 0 10 μ 20 μ



Fig. 16. 0 10 μ 20 μ



Fig. 14. 0 10 μ 20 μ

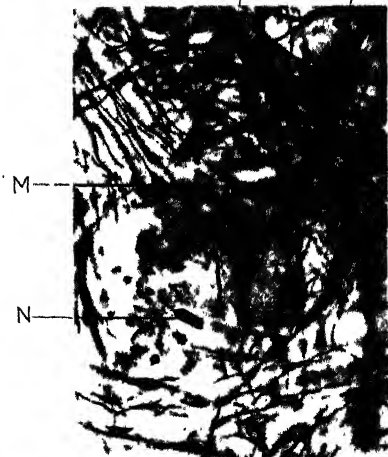


Fig. 15. 0 10 μ 20 μ 30 μ

Marui (1918) found terminals on Mauthner cells in his studies on the trout, but recognised the "end feet" as points where the dissolution of fibres occurred. Although there may or may not be specialised terminals on the nerve cells of fish, these criticisms have no bearing on present studies, where care has been taken to avoid post-mortem changes, while detailed examination with the binocular microscope revealed undoubted sharply stained loops at the ends of the finest nerve fibres.

Granted that they exist, it may be questioned whether the boutons are nervous in nature. The points in favour of this view are the fact that they are demonstrated by staining methods specific for axis cylinders, and that they occur only at terminals of nerve fibres, or in the case of *boutons de passage*, along the length of nerve fibres. Finally, as these investigations show, the boutons degenerate when nerve fibres are cut.

Assuming that the boutons are actual nervous structures, it may be worth while to consider whether they are really synapses. The present studies confirm the observation of Cajal that the boutons end freely upon the cells and that there is no protoplasmic continuity between them and the intracellular neurofibrils as Held believed. In instances where the cell-surface shrinks, many boutons pull away quite readily and there is no evidence at all of any process proceeding from them to the neurofibrils. Also, it is significant that degeneration following section of the afferent nerves does not extend beyond the bouton into the cell. Finally, that the boutons are the synapses is strongly suggested by the work of Windle (1930), showing a close correlation between the increase in the numbers of these terminals in growing kittens and the complexity of the animals' reflex behaviour.

The observation that boutons, when their nerve supply is severed, show characteristic changes in 2-3 days suggests a new method of following nerves to their terminus. This method has the advantage of giving results in a much shorter time than the older neuro-anatomical methods; and, further, demonstrates the actual point of termination of the fibre or tract, which was not possible before. Preliminary experiments to trace the endings of the afferent tracts to the cerebellum, and the termination of the dorsal columns of the white matter of the cord in the gracilis and cuneatus nuclei, have already proved successful. The chief result of the application of the method to the study of the spinal cord has been, as previously stated, the observation that degenerating boutons are found on cells in the mid-region of the grey matter following de-afferentation of the same side. It is suggested, therefore, that afferent fibres which terminate locally do so by means of *boutons terminaux*.

around cells of this region. Thus, the influence of afferent fibres on ventral horn cells or cells of the opposite side must involve additional neurones. This suggestion is represented diagrammatically in fig. 4B.

The author wishes to express his thanks to Professor Sir Charles Sherrington, Dr. H. M. Carleton and Dr. J. C. Eccles for their helpful advice during this work; likewise to his brother, Mr. H. E. Hoff, for his assistance in the operations. He is also indebted to Professor E. S. Goodrich for the use of his microphotographic apparatus.

VIII. *Summary.*

(1) This paper is the report of investigations concerning the synapses in the spinal cord of the cat. The small loops or "boutons" which occur on the cell and dendrite surfaces, either as terminals of nerve fibrils or along their courses, are regarded as interconnections or synapses between neurons. These studies support this view.

(2) A method of fixation by injecting chloral hydrate into the living, anæsthetised cat, preparatory to staining with Cajal's method, is described. This gives consistent and equable impregnation in which all the *boutons terminaux* and *boutons de passage* are stained.

(3) The histology, distribution and quantitative occurrence of boutons are described.

(4) It is shown that after section of the afferent roots of the nerves of the lumbar or cervical enlargements in the adult cat, the boutons upon some of the cells degenerate. This degeneration is limited to boutons on the cell-bodies and dendrites in a definite region in the medial part of the grey matter on the de-afferented side. Twenty-four hours after the operation, such degenerating boutons can be recognised by their slightly swollen appearance. Their disappearance is complete in 4 to 6 days, being preceded by further swelling, elongation and granulation.

(5) It is suggested that this observation may furnish a method for tracing the terminations of nerve fibres in the central nervous system. Further applications of this method are being pursued.

(6) The boutons of cells in all other regions of the grey matter in operated animals are normal. It is therefore concluded that there is no direct termination of afferent fibres on ventral horn cells of the same side, nor any direct crossing to the opposite side.

EXPLANATION OF PLATES.

PLATE 8.

- FIG. 7.—A spinal cord cell showing normal *boutons terminaux* and *boutons de passage*. The cell is situated in the lateral part of the mid-region of the grey matter, on the left side, in the fifth cervical segment of an adult cat's cord. Five of the right afferent roots in the cervical enlargement had been cut 4 days before the animal was killed; and in the same section, enlarged, granular, degenerated boutons could be seen on the right side. Cat 21, operation 28. Magnification, 1371 diameters.
- FIG. 8.—A bouton on a small cell near the ventral commissure on the operated side of the cord of a cat killed 6 days after complete de-afferentation of the right side of the lumbar enlargement. The bouton measures $3\mu \times 3\mu$, exclusive of a small forked process which can be seen coming from its right side. There is no continuity between the process and the neurofibrils which can be seen within the cell. Fifth post-thoracic segment. Cat 4, operation 4. Magnification, 1371 diameters.
- FIG. 9.—A normal bouton ending on the proximal part of a large dendrite of a funicular cell. Normal adult cat. Magnification, 1371 diameters.
- FIG. 10.—A bouton on a cell in the mid-region on the operated side of the grey matter. The cat had been killed 29 hours after de-afferenting the entire lumbar enlargement on the right side. The loop is slightly swollen, and the tiny internal fibril with its moniliform swelling is seen. Seventh post-thoracic segment. Cat 15, operation 25. Magnification, 1414 diameters.
- FIG. 11.—A normal bouton on a funicular cell of the lumbar enlargement, showing the tiny filament sometimes seen within a normal terminal. All other boutons are out of focus. The normal appearance of the cell-body with the silver stain is shown. Magnification, 1371 diameters.

PLATE 9.

- FIG. 12.—Degenerating terminals on a cell of the same section as the bouton of fig. 10. Normal *boutons terminaux* are indicated by N. Seventh post-thoracic segment. Cat 15, operation 25. Magnification, 1414 diameters.
- FIG. 13.—A degenerate terminal from the cord of a cat killed $2\frac{1}{2}$ days after a medial longitudinal incision of the cord from the third to the sixth post-thoracic segments. The section is taken from the fourth post-thoracic segment. Cat 7, operation 8. Magnification, 1698 diameters.
- FIG. 14.—Boutons which have degenerated 43 hours. The cell illustrated occurs in the sixth post-thoracic segment on the operated side of the cord in a cat whose right fifth, sixth, seventh and eighth post-thoracic afferent nerves were severed central to the ganglia. The microscope was focussed to show particularly the boutons on the proximal parts of the dendrites. Cat 5, operation 6. Magnification, 1414 diameters.
- FIG. 15.—Boutons of a cat killed 3 days after section of the right fifth, sixth, seventh and eighth post-thoracic afferent nerves. The microscope was focussed on two large, degenerate terminals, one pear-shaped (M) and the other long and spatulate (N). Three normal terminals are seen between and slightly to the left of these two. The cell-body is not in focus. Cat 2, operation 2. Magnification, 709 diameters.
- FIG. 16.—Part of the same cell as shown in fig. 15. The boutons have become greatly enlarged, elongated and granular. Two normal boutons are also in focus. Sixth post-thoracic segment. Cat 2, operation 2. Magnification, 1414 diameters.

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*The Recent Development of Melanism in the Larvæ of certain Species
 of Lepidoptera, with an Account of its inheritance in Selenia
 Bilunaria Esp.*

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I.—INTRODUCTORY.

One of the most remarkable evolutionary processes, the more striking since it has occurred before our eyes, has been the rise and spread of melanism and melanochoism amongst the Lepidoptera. Commencing about 1850 in the Manchester area in England with the Geometrid moth *Amphidasys betularia* L., which yielded the black form *carbonaria* Jord. (*doubledayaria* Mill.), this development has proceeded so rapidly, and become so widespread, that now there is scarcely a country in Northern and Central Europe which does not produce its quota of melanic insects. Moreover, the same state of affairs exists in the North-Eastern United States, although there the number of species affected, up to the present, is not so great as in Europe. Another important feature about these changes lies in the circumstance that, almost uniformly,

in Europe and in the United States, the first species to exhibit melanism in any given area have been *Amphidasys betularia** and *Tephrosia crepuscularia*.

From the beginning, the Geometridæ, more especially the subfamily Boarmiinæ, have provided not only the bulk of the melanic varieties, but also the greatest numbers of individuals. In many areas, as for example in the case of *A. betularia* and *Ypsipetes trifasciata*, only black examples occur. Nevertheless, other groups include species which have gone black; for instance, the Noctuidæ present black forms of *Aplecta nebulosa* Hufn., the Cymatophoridæ of *Cymatophora* or F., the Arctiidæ of *Spilosoma lubricipeda* L., the Gelechiidæ of *Chimabacche fagella* F., and so on.

Nor must it be imagined that the impulse is spent; far from it, new species involved, and new melanic forms arising, are constantly being reported by investigators. However, both entomologist and geneticist alike have hitherto, for the most part, been silent concerning a further evolutionary movement. In spite of that, it is no longer correct to assume that only the perfect insects of the various species display melanistic tendencies. Recently, more particularly since 1918, an apparently new phase has been ushered in; black larvæ, representative of quite a large number of species, have been detected in the course of my researches.

Of the occurrence of black larvæ of *Abraxas grossulariata* L. and of *Miselia oxyacanthæ* L., I have had knowledge for a considerable number of years, and as long as the phenomenon was restricted to these, little was done but to draw the attention of other workers to the fact. When, however, additional species yielded larvæ of the same coloration, I not only redoubled my efforts, in order, if possible, to detect species during the period of transition but, in addition, in the case of certain species instituted experiments as to its inheritance and its dependence or otherwise on the environment.

The progress of the new movement has been so rapid that it is proposed (1) to place on record, without delay, the present position in respect to the incidence of larval melanism in Northumberland and Durham, and (2) to describe experiments carried out on melanic and other larvæ of *Selenia bilunaria*. By so doing, it is hoped that workers elsewhere will be stimulated to make observations so that facts of first-class importance may be amassed, before it is too late, concerning the rate of the spread of the phenomenon, and the order in which various districts and countries are affected. In this way the solution of the general problem of melanism may be approached, if not completely gained.

* In the United States in the form of its representative *Amphidasys cognataria* Gn.

II.—A CONSIDERATION OF THE SPECIES EXHIBITING LARVAL
MELANISM.*Family Geometridæ : Subfamily Boarmiince.*

*Abraxas grossulariata** L. (1895)†.—Of the existence of black larvæ, as well as of black pupæ, of this species on Tyneside I have had knowledge for many years, and very early indeed such larvæ were supplied to the Rev. G. H. Raynor and other workers with *Abraxas*. In this area, Birtley, N. Durham, a manufacturing and mining district, $5\frac{1}{2}$ miles south of Newcastle-upon-Tyne, they occur commonly in all old-established gardens well stocked with members of the genus *Ribes*, *i.e.*, with currants and gooseberries, the foliage of which constitutes their favourite food. On the dark bark of these shrubs the larvæ rest by day, where the striking reds and whites of the typical larvæ make them very conspicuous objects indeed, whilst the black forms assimilate closely to their surroundings. This, in the absence of attack by birds, seems of little significance.

In addition to these garden habitats, two colonies on sloe (*Prunus spinosa*) were known to me and both provided typical larvæ only, but the colonies were recently destroyed by building operations. Of these colonies, one was an outlier, half a mile north of the nearest colony with melanics, and, so far as I know, remote from others to the north, east or west, whilst the other was within the melanic area.

When I first collected black larvæ from affected habitats, specimens turned up singly. Subsequently, they gradually increased in numbers until at present the percentage in our garden oscillates between 50 and 60—a level maintained for years.

Although the melanics are generally sharply marked off from typical forms, intermediates do occur, and these lean strongly to the melanic side. As a result of the latter peculiarity, the experiments dealing with the inheritance of larval pigmentation are not quite complete. Still, the results seem to indicate that melanism comports itself as a Mendelian dominant.

Contrary to expectation, no connection exists between black larvæ and black moths. In general, although I once bred var. *hazeleighensis* from a black larva, such larvæ give rise to typical imagines only.

Opisthograptis luteolata L. (1930).—The larval pigmentation of this species was discussed long ago by Poulton and other workers, and shown to be influenced

* An asterisk denotes that the species possesses melanic varieties in the imagines also.

† The date supplies the year in which I first detected melanism in the larva.

by the surroundings. In spite of that, no melanic forms have been reported previously.

With us the larvæ feed on hawthorn, mountain ash, white beam, sloe, birch, etc., when usually, depending on the environment, the larvæ exhibit green or purplish brown colorations. Black forms now occur at Birtley on hawthorn.

*Tephrosia bistortata** Goeze (1919).—Larvæ bred from larch-feeding parents, taken in Chopwell Woods, Co. Durham, give larvæ very unlike the pale greyish larvæ beaten from birch on the Culbin Sands, Elgin. They include variegated individuals, ranging in extreme cases to an intense black.

*T. crepuscularia** Hb. (?).—The latter statement holds good of broods of this near ally of *T. bistortata* from York.

*Boarmia repandata** L. (1920).—In this species we are concerned with an insect which, once very common, seems to be diminishing in numbers in the manufacturing areas on Tyneside. From 1902 to 1914, larvæ, collected chiefly from hawthorn, were of a greyish putty hue, and the same holds true still of those obtained in the wooded districts of the Derwent Valley. Commencing with larvæ taken from rose and hawthorn near Birtley, a gradual change has resulted in the displacement of the paler forms by black and smoky coloured individuals. Last year, of the small number I secured, all were melanic. Here, although no connection exists between black larvæ and black moths, black larvæ yield melanic and melanochoic imagines; but so do some of the pale larvæ!

*Boarmia gemmaria** Brh. (1907).—This insect feeds chiefly on ivy in the Newcastle district, and can be found in any street in our towns where that plant is cultivated. In the country, hawthorn, sloe, etc., are favoured foods, and there the larvæ are slaty grey. In Gateshead and elsewhere, melanic larvæ are quite common. All of our larvæ give rise to melanochoic moths.

Hybernia aurantiaria Esp. (1931).—Normally, this larvæ abounds on sycamore in these districts and is then easy to recognise from its characteristic pale dorsal markings. Last year, when obtaining larvæ from hawthorn, just west of Birtley, for cytological purposes, I found that many were quite black. So great a danger arose of confusing them with black examples of *H. marginaria* that they had to be discarded.

*Hybernia defoliaria** Cl. (1919).—This larva is found almost without exception on oak here, and although dark grey in the ground colour, looks a very conspicuous object with its brownish markings and bright yellow spiracular stripe. In the Team Valley, N. Durham, where it is distinctly rare, black forms now

occur and form about 10 per cent. of the population. Some specimens should be described as melanochoic rather than melanic.

*Hybernia marginaria** Bkh. (1910).—This species abounds on hawthorn, birch, larch, elm, etc. Near the town of Birtley, brownish individuals tending to smoky, are common on hawthorn, whilst in the Brooms Wood, in a deep, shady, damp ravine, facing north, black larvæ occur on birch and elm to the extent of 40 per cent. Similarly, at many points where the insect deserts birch in favour of larch, the bulk of the specimens beaten are quite black. This tendency for larvæ to become melanic when they adopt a larch diet, occurs in many species, and even the imagines, under the same circumstances, are melanochoic (Harrison, 1927).

*Phigalia pedaria** F. (1918).—This is another species in which the pigmentation of the larvæ generally depends on the colour of the surrounding surfaces. In spite of this, in Chopwell Woods and elsewhere, wholly black varieties are to be found on larch, the coloration of which does not rely on environmental control. Similar black examples turn up not infrequently in other stations in the manufacturing zones on oak, hawthorn, and birch.

*Amphidasys betularia** L. (1930).—In this insect we are dealing with one investigated by Poulton (1884, 1885, 1892) in his classical work on the influence of environmental factors on larval colours. Most commonly, owing to the fact that in these areas the chief foods are birch and hawthorn, purplish brown larvæ prevail; rarely, however, on oak, elm, willow and ash, greenish forms crop up. Two years ago, in Long Acre Dene Wood, about 5 per cent. of the individuals from oak were jet black (Harrison, 1931). Experiments, still proceeding, prove that these larvæ are genetically black, and that their surroundings cannot influence them. Only black imagines occur in this wood.

Ellopija prosapiaria L. (1925).—In this case, so far as my knowledge goes, the larvæ, which feed on *Pinus sylvestris*, do not respond readily to the environment. Structurally and in colour, despite that fact, they assimilate closely to the pine twigs upon which they rest by day. The pines in Dipton Woods, Northumberland, produce a few coal-black examples annually.

*Selenia bilunaria** Esp. (1928).—Observation has been kept on this species locally, for various genetical purposes since 1903, and every year, whenever possible, supplies of larvæ have been obtained.

With us, the larvæ possess a wide range of food plants, birch, hawthorn, alder, oak, blackthorn, willow, rose, bramble, meadowsweet, although they prefer the first three.

Arising from the ready response to their surroundings, hawthorn forms are quite pale when beaten wild ; on the contrary, wild larvæ from birch and alder are usually of a dull plum colour. Wholly black larvæ were obtained from an alder "car" on Waldrige Fell, Co. Durham, a locality in which the imagines of many species are melanic. Nevertheless, in the case of *S. bilunaria*, black larvæ yield typical insects. The only black imagines known locally, and only detected recently, were bred from pale hawthorn larvæ from Chopwell, Co. Durham.

Work carried out on this species is described later in this paper and should be consulted for facts concerning inheritance, etc.

Selenia lunaria Schiff. (1931).—Here we are dealing with a species much rarer than its congener and only beaten by me from oak and birch. In Long Acre Dene Wood, a wood in which much of the cryptogamic life has been destroyed by Gateshead smoke, two coal-black larvæ were obtained last year from the same oaks as provided the black larvæ of *Amphidasys betularia*. In addition to displaying this unwonted garb, they appeared more strongly humped than usual.

Similarly, on the Culbin Sands, Forres, Elgin, I secured 63 larvæ of the species, utterly unlike typical forms, from rugged, windswept birches. In their case, the humps were, to a great extent, suppressed, the incisions accentuated, and the colouring melanochoic. As demonstrated by Poulton, typical larvæ tend to approximate the hues of the surfaces near which they rest.

Hygrochroa syringaria L. (1931).—This insect was only discovered in quantity in the two north-eastern counties in 1929. In its wild condition its food plant is always *Lonicera periclymenum*, and on that plant its pigmentation much depends on the openness of the wood which it frequents. At Wylam, Northumberland, in a very sunny wood on the river bank facing south, the larvæ tend to be pale grey, whilst at Styford, Chopwell and Gibside, in darker and denser woods, the prevailing hues are brown purple. Melanic larvæ have only occurred at Chopwell.

Himera pennaria L. (1926).—This species does not turn up very near to Newcastle but, nevertheless, it approaches the smoke zone at Consett. It usually feeds upon *Quercus*, *Ulmus*, *Alnus* and *Calluna*, and the larvæ react slightly to the environment. Black forms of the larvæ have been found at Corbridge on alders growing along the Devil's Water.

Ennomos alniaria L. (1931).—This larva I used to beat from birch and alder on the Culbin Sands every year from 1906 to 1912. From the latter year until 1931 that locality was not visited. However, last year, of 28 larvæ obtained

from birches, three were quite black and strongly resembled those of the American species *E. subsignarius*. The ordinary larva of this species is sensitive to its environmental conditions.

*Gonodontis bidentata** L. (1912).—Larvæ of this species, under ordinary circumstances of a dull, putty colour, adapt themselves readily to their resting surfaces. On lichen-clad birches, for instance, beautiful black and green forms, harmonising well with the hues of various species of *Parmelia* are developed, whilst on birches free from these epiphytes the larvæ are usually brownish. Similarly, on larch they become melanochroic with rusty markings, but an occasional black larva is encountered.

In towns and urban areas, where ivy and privet provide the food, the larvæ darken and here specimens, genotypically and phenotypically black, are quite frequent. Experiments are in progress with this species.

Crocallis elinguaris L. (1921).—The larvæ, again light sensitive, are of an ochreous brown in the ground colour and feed on blackthorn, hawthorn, hazel, rose, etc.; pure black forms occur on scrubby *Cratægus*, near Birtley, Co. Durham.

Family Noctuidæ : Subfamily Trifinæ.

Miselia oxyacanthæ L. (1905).—In this insect we break away from the Geometridæ and come to the only species amongst the Noctuidæ in which true melanic larvæ occur with us. In the Team Valley area, although the usual fuscous larvæ can still be obtained, black varieties may form up to 80 per cent. of the population, although this figure varies from colony to colony. Experiments have been undertaken on the inheritance of melanism in the larvæ of this species, but it is so slow-breeding that they cannot reach fruition for some time. As far as they go they afford evidence that larval melanism in *Miselia oxyacanthæ* is a Mendelian dominant.

III.—EXPERIMENTS WITH LARVÆ OF *SELENIA BILUNARIA*.

(a) *To Determine the Influence, if any, of the Environment on such Larvæ.*

Experiment 1.—Four of the usual 3½-inch glass-topped tin boxes used for larva feeding purposes were taken. Into one of these were placed thin twigs of hawthorn, and into the other three similar twigs mixed with spills of dark brown paper. The first box (batch A) and one of the second type of box (batch B) were each provided with 30 larvæ reared from parents which as larvæ had belonged to the pale grey-brown coloured hawthorn form from Chopwell; in the third were inserted 30 larvæ (batch C) from parents bred from brown purple larvæ from alders on Waldrige Fell, whilst the last (batch D)

was supplied with 15 Chopwell larvæ and 15 reared from black Walldridge alder larvæ.

At the expiration of a fortnight, the larvæ were transferred to four of the glass cylindrical cages usually employed for my cultures and there continued on a hawthorn diet, care being taken to keep all four cages under similar conditions externally.

Of these cages, that containing batch A was left untouched ; in the case of the whole of the others the base and cover were painted brown, whilst amongst the foliage in each were placed nine wooden rods of the same size as a lead pencil, likewise painted brown. Inside the same three, on the dark side, two slabs of wood, 6 inches by $2\frac{1}{2}$ inches by $\frac{1}{4}$ inch, also brown in colour, were inserted so as to reflect as much light as possible amongst the foliage. The larvæ were kept under these conditions until just about to spin, when the state of each batch in respect to colour was carefully determined.

Batch A.—Under ordinary cage conditions, with food similar to that to which the larvæ had been accustomed under natural conditions, it has been found that little movement in pigmentation occurs. The evidence yielded by this batch proved no exception ; the larvæ repeated the conditions of wild lots from Chopwell hawthorn colonies in their swing of variation.

Batch B.—Here, in spite of the occurrence of a definite range in variation, the tendency of *Selenia bilunaria* larvæ to adjust themselves to the environment was manifested unmistakably. They were all darker and browner, with much less inclination to ochreous or grey than either their parents or batch A, which acted as control.

Batch C.—In this instance the larvæ, influenced by the prevalent brown tones of the cage fittings, resembled wild alder* larvæ in colour and in the comparatively slight amount of variation.

Batch D.—At the end of the experiment there remained 26 larvæ. Of these 14 were quite black and 12 dark brown or dark brown purple.

Clearly the black larvæ are quite independent of their surroundings for their coloration, whilst the larvæ accompanying them had been influenced by the colours of cover, base, pencils and slabs.

An additional factor, however, must be taken into account. Throughout their lives in the cages, the typical larvæ had rested on twigs in close proximity to larvæ which were black in every instance. As a result of this circumstance, the environmental conditions were distinctly darker in tone than in the case of batch B, in which the general furnishings of the cage were the same. To

* Except for melanic alder larvæ, Walldridge birch and alder lots agree in coloration.

that fact the somewhat darker tone of the non-melanic larvæ in this case was, no doubt, due.

Experiment 2.—In this test two glass-topped tin boxes were lined with white paper and supplied with hawthorn twigs mingled with spills of white paper. Into the first 30 Walldridge larvæ (batch E) from parents homozygous for brown purple in the larvæ condition were put, and into the second 15 similar larvæ and 15 derived from Walldridge "blacks" (batch F). Once more, at the end of 14 days, the larvæ were transferred to hawthorn in glass cages furnished like those of batches B, C and D, except that the fittings were painted with white enamel paint instead of brown.

When about to spin, the larvæ were examined as before, again with interesting results. In the case of batch E the aspect of the larvæ was very remarkable; 8 were of such a pale grey as to be classified best as whitish, 15 were various tones of light rusty brown and 4 were somewhat darker; none reached the tone level of batch C (regarded as controls) or of wild stocks. Batch F, as far as possible, repeated the circumstances of the preceding lot. Five larvæ were exceedingly pale, 6 came into the same class as the second section of batch E, so that the presence of the black larvæ in this lot had been almost, if not quite, negligible. The 10 remaining larvæ were wholly black. Thus the indications of this experiment* agree with those of experiment 1.

Experiment 3.—Two glass-topped tin boxes were employed; the first was provided with 30 ordinary Walldridge larvæ and the second with 30 larvæ from the black larva producing strain, both lots being fed on birch. After 14 days they were removed to the usual cylindrical cages and the birch diet continued. As a result both batches when full-grown repeated parental larval conditions.

. As determined by all three lines of approach it appears certain that melanism in black *Selenia bilunaria* larvæ is germinally fixed and cannot be altered in response to the environmental stimuli brought into play in the experiments just described.

(b) *To Determine the Mode of Inheritance of the Larval Melanism of Selenia bilunaria.*

In this section of the work a southern race of *Selenia bilunaria*, yielding rich yellow-brown, variegated larvæ under cage conditions with me, was

* Another remarkable observation was made in these two tests. In some way or other the white surroundings seem to prolong the duration of larval life enormously; this matter is being further investigated.

employed in conjunction with Walldridge and Chopwell races, producing black and normal larvæ respectively. As the northern strains are univoltine and those from the south bivoltine, the two sets do not synchronise in times of emerging from the pupæ. Hence, part of the Walldridge pupæ had to be forced gently. By this procedure, it was possible to place reciprocal pairs of Walldridge and southern insects in the muslin pairing cages, and 6 weeks later to treat Walldridge and Chopwell insects in the same fashion.

In all the matings fertile eggs were secured which duly hatched, to give rise to larvæ which were reared under conditions described in experiment 3 above. Under this treatment, the F_1 lots were brought to maturity with results, as far as larval pigmentation is concerned, set out in Table I.

Table I.—Homozygous Type (Larva) \times Homozygous Melanic (Larva)
TT \times tt.

Family.	Origin of family.	Types.*	Melanics.*
A	Chop. ♀ \times Wald. ♂	43	0
B	Wald. ♀ \times Chop. ♂	76	0
C	Surrey ♀ \times Wald. ♂	48	0
D	Wald. ♀ \times Surrey ♂	91	0
	Actually reared	258	0
	Theoretical result	258	0

* As larvæ, of course.

The data just displayed suggest that, whether the form with pale larvæ originates at Chopwell or in the south, larval melanism in *S. bilunaria* is recessive.

At this stage, owing to the bivoltine nature of the southern insects and the uncertainty of the times of eclosion of the F_1 batches involving them, the work, to a considerable extent, was concentrated on the Chopwell-Walldridge lots.

Insects taken from the latter cultures were then paired *inter se*, back-crossed on homozygous types from Chopwell, and on moths from black Walldridge larvæ. In addition, such matings as were possible with the parallel southern lots were brought about. Ova were duly deposited, and larvæ reared as before. The facts concerning the coloration of the latter appear in Tables II, III and IV.

In these tables we have confirmed the suggestion of the F_1 batches that melanism in *S. bilunaria* larvæ behaves as a simple Mendelian recessive for, within the limits of experimental error, the expected 3 : 1 and 1 : 1 ratios of

Table II.— F_2 Generations from Families A, B, C, and D. $Tt \times Tt$.

Family.	Description of family.	Types.	Melanics.
E	F_2 A	53	12
F	F_2 A	129	44
G	F_2 B	103	32
H	F_2 C	68	22
I	$A \varphi \times D \sigma$	45	14
	Actual result	398	124
	Theoretical result	391.5	130.5

Table III.— F_1 (ex A, B, C and D) \times Homozygous Types (Chopwell). $Tt \times TT$.

Family.	Origin of family.	Types.	Melanics.
J	$A \varphi \times \text{Chop. } \sigma$	113	0
K	$B \varphi \times \text{Chop. } \sigma$	74	0
L	$\text{Chop. } \varphi \times C \sigma$	95	0
M	$D \varphi \times \text{Chop. } \sigma$	77	0
	Actual result	359	0
	Theoretical result	359	0

Table IV.— F_1 Insects ex A, B, C and D \times Homozygous Melanics (Waldridge). $Tt \times tt$.

Family.	Origin of family.	Type.	Melanics.
N	$\text{Wald. } \varphi \times A \sigma$	49	53
O	$\text{Wald. } \varphi \times B \sigma$	56	51
P	$B \varphi \times \text{Wald. } \sigma$	39	34
Q	$B \varphi \times \text{Wald. } \sigma$	48	50
R	$\text{Wald. } \varphi \times B \sigma$	29	23
	Actual result	221	211
	Theoretical result	216	216

pale larvæ to melanics were obtained in the F_2 generation and in the back cross with the recessive parent, whilst, as was anticipated, the other type of back cross yielded larvæ wholly of paler proclivities.

Only two further types of mating remain for consideration, and these are (1) that between homozygous melanics and (2) that between homozygous

types. Since the outcome of such pairings always agrees with expectation, no matter whence the strains originated, the facts they afford are massed without further comment in Tables V and VI.

Table V.—Homozygous Black \times Homozygous Black.

$tt \times tt$.

Family.	Origin of family.	Types.	Melanics.
S	E ♀ \times F ♂	0	57
T	F ♀ \times E ♂	0	61
U	Wild	0	43
	Actual result	0	161
	Theoretical result	0	161

Table VI.—Homozygous Type \times Homozygous Type.

$TT \times TT$.

Family.	Origin of family.	Types.	Melanics.
V	Waldridge	89	0
W	Saxony	76	0
X	Chopwell	112	0
Y	Beamish	54	0
Z	Elcheester	129	0
	Actual result	460	0
	Theoretical result	460	0

Summing up the results of the two lines of enquiry, we are entitled to conclude (1) that larval melanism in *Selenia bilunaria* is germinally fixed, and (2) that it has been proved to behave in inheritance as a simple Mendelian recessive.

Summary.

(1) Recently, under natural conditions, certain lepidopterous species have developed a melanic form of their larvæ.

(2) This melanism is germinally fixed and does not depend on the environment for its expression.

(3) The species affected, with one exception, belong to the Geometridæ, and in that family to the subfamily Boarmiinæ.

(4) Very many, if not all, of the same series of species possess larvæ with

the power of adapting their coloration, and even structure, to their immediate environment.

(5) The single species not belonging to the Geometridæ is the Noctuid, *Miselia oxyacanthæ*.

(6) Almost without exception, the larvæ discussed feed on trees and shrubs and rest by day on the twigs and bark.

(7) As determined by the extermination of lichens, very many of the melanic forms were first detected within the sphere of influence of smoke from Tyneside factories.

(8) It is not asserted that the smoke is the cause of the new departure ; the fact is pointed out for information.

(9) In *Selenia bilunaria* the melanism is inherited as a Mendelian recessive.

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The Combination Curves, Hydrogen Ion Regulating Powers and Equivalent of Serum Albumin.

By E. B. R. PRIDEAUX and D. E. WOODS.

(Communicated by F. S. Kipping, F.R.S.—Received April 21, 1932.)

The acid, alkali combination curve of serum albumin has received less attention than those of gelatin and ovalbumin, which formed the subject of previous papers, Prideaux (1931) and Prideaux and Woods (1932).

In order to obtain data for the comparison of the two albumins, it was necessary to determine the α , p_H points throughout the whole range. From these, the buffering powers, and also the equivalent weights at various points were calculated as before. A close similarity to ovalbumin was expected, and on the whole, found, but definite differences in the forms of the curves and the buffering powers were noted. Serum albumin appeared to be a more robust protein, and in particular had a greater stability in the presence of alkali.

Experimental.

The serum albumin was prepared from ox serum which was purified by the method of Goignier and Pauli (1931). It was recrystallised three times, dialysed against distilled water for 8 weeks until the conductivity fell to 0.84×10^{-4} mhos. at 25° , then subjected to electro dialysis for a further 2 days which reduced the conductivity to 0.52×10^{-4} . The ash content was 0.008 gm. per 1 gm. of the dry product.

The albumin contents of the stock solutions were tested in the manner already described, Prideaux and Woods (1932).

The measurements with the hydrogen electrode and the calculation of the results also followed the same course.

The results are summarised in the following tables.

In Table I, one out of three concordant series is given in each case.

The Comparison of Ovalbumin and Serum Albumin.

The combination curves of the two albumins bear a close resemblance to one another. The p_H of serum albumin, however, is the more regular and less affected by those fluctuations which appear in the curves of ovalbumin and also of gelatin in the neighbourhood of p_H 7 to 9.

Table I.—The Titration of 1 g. of serum albumin with 0.1 N acid or alkali of which x c.c. are required to bring 100 c.c. of 1.0 per cent. or 0.1 per cent. solution from pI to p_H at 25° C. In cases where a blank correction was introduced, the original values are given in brackets.

1 per cent. albumin.				0.1 per cent. albumin.			
Acid.		Alkali.		Acid.		Alkali.	
x .	p_H .	x .	p_H .	x .	p_H .	x .	p_H .
0.75	4.3	0.8	5.4	1.0	4.35	1.0	5.6
1.43 (1.44)	4.05	1.37	5.9	2.0	4.0	2.0	6.5
2.0 (2.16)	3.9	1.97	6.4	2.76 (3.0)	3.8	3.0	7.35
2.35 (2.6)	3.75	2.75	7.4	3.69 (4.0)	3.65	4.0	8.0
3.26 (3.64)	3.5	3.35	8.0	4.55 (5.0)	3.45	5.0	8.4
4.52 (5.1)	3.3	3.95	8.45	6.45 (7.0)	3.35	6.0	8.65
5.43 (6.07)	3.25	4.7	9.0	8.75 (10.0)	3.0	7.0	8.85
6.5 (7.77)	3.0	5.0	9.2	11.2 (13.0)	2.0	9.0	9.2
7.1 (9.0)	2.85	5.48	9.35			11.0	9.4
7.45 (9.71)	2.75	6.13	9.6			12.0	9.75
8.16 (11.16)	2.65	6.48	9.8			14.85 (15.0)	10.0
8.25 (12.86)	2.45	6.95 (7.0)	10.0				
8.76 (18.0)	2.2	7.45 (7.62)	10.25				
		8.6 (8.82)	10.5				
8.5 (20.0)	2.1 (2.1)*	9.25 (10.0)	10.7				
9.2 (24.0)	2.0 (2.1)*	10.05 (10.83)	10.8				
9.3 (33.3)	1.8 (2.0)*	10.54 (12.0)	11.0				
		11.96 (14.6)	11.3				
		12.7 (16.0)	11.4				
		13.35 (17.45)	11.5				
		14.55 (19.8)	11.6				

* Colorimetric, with methyl violet or thymol blue.

There are, as usual, three main branches. The acid branch extends from slightly below p_H 3.0 to about p_H 4.0 as a straight line, thence with a slightly increasing slope to p_H 5.0, and then through the inflection, the centre of which is at $p_H = 7$. The slope of the curve then gradually diminishes to about p_H 10, from which point it becomes a straight line to $p_H = 12.6$ and beyond.

In contrast to ovalbumin, a slight inflection is found in the curve of serum albumin on the extreme acid side, when p_H has fallen below 2.6. These values were checked colorimetrically by the use of thymol blue and methyl violet against standard solutions containing HCl and KCl. The colorimetric values, corrected approximately for protein error by subtracting 0.53 and 0.19

respectively, agreed sufficiently well with the electrometric. The last three values of Table I, bracketed after column 3, were obtained in this manner.

In discussing numerical constants it is necessary to distinguish between the assumption that the protein in pure water is mainly present as undissociated molecules (M) or as ampholyte ion (A).

An equivalent weight by titration may be deduced from the slight inflection at about $p_H = 2.2$. This corresponds to the saturation of the free basic groups (M), which occurs after the addition of 8.7 ± 0.2 c.c. of 0.1 N HCl to 1 gm. of serum albumin as a 1 per cent. solution.

The equivalent as base (M) by titration is therefore 1150 , the corresponding value for *gelatin* being 1180 . In view of the lowness of p_H and magnitude of the acid blank correction, the difference between these values has no significance. On account of the absence of an inflection it is impossible to state a lower limit to the basic titration equivalent of *ovalbumin*. The equivalent weight of this in the acid range, however, is certainly lower, *i.e.*, it has the greater acid fixing power. For nearly 10 c.c. of 0.1 N acid are required to bring ovalbumin, and only about 8 c.c. to bring serum albumin, from pI to $p_H = 2.6$. Such a large difference might well be used in estimating a mixture of these proteins.

As in the preceding papers, the buffering power $B = \Delta C / \Delta p_H$ (C = equivalents of acid or alkali to 1 gm. of albumin) will be defined by the cotangent of the curve ($= 10^4 B$), where this is linear over a moderate range. Also the apparent equivalent of the acids or bases which are functioning over this range is $0.576/B$.

Table II.—Comparison of the Buffering Power against Acids of Ovalbumin and Serum Albumin.

Ovalbumin.		Serum albumin.		Gelatin.	
p_H range.	$10^4 B$.	p_H range.	$10^4 B$.	p_H range.	$10^4 B$.
2.6–4.4	5.1	2.6–4.4	4.4	—	—
3.0–5.0	3.5	3.0–5.0	3.25	3.0–5.0	3.9
3.0–4.0	4.7	3.0–4.0	4.2	—	—
4.0–4.7	2.6	4.0–4.7	2.7	—	—
4.7–6.0	1.7	4.7–6.0	1.25	—	—

As mentioned in the preceding paper, the comparison between albumin and gelatin is not perfect, on account of the difference in the type of the curves.

The equivalents calculated from the straight line 3.0-4.0 are 1225 ovalbumin and 1375 serum albumin. It will be noticed that the equivalent 1370 is somewhat higher than that calculated from the titration, 1150. This shows that new basic groups (M) which do not function between 3 and 4, come into action below 3.

A comparison of the inflection on the alkaline side shows that gelatin has a much lower buffering power, besides showing a second inflection at about $p_H = 12$, which is not found in the curves of the albumins.

Table III.—Comparison of the Buffering Power against Alkalies of Ovalbumin and Serum Albumin.

Ovalbumin.		Serum albumin.		Gelatin.	
p_H range.	10^4 B.	p_H range.	10^4 B.	p_H range.	10^4 B.
6.0-8.0	0.75	6.0-8.0	0.75	6.0-8.0	0.44
9.0-10.5	curvature changing	9.0-10.5	3.07	—	—
—	—	10.0-11.0	4.0	9.8-11.2	1.9
10.3-11.3	4.0	(mean) 10.4-11.6	5.8	—	—

The two albumins behave identically round the point of absolute neutrality, where the buffering power is at a minimum. That of gelatin is even lower here. The regulating power is recovered with increasing p_H , although at slightly different rates in the two cases. So far as a comparison is possible, it would appear that serum albumin has the higher buffering power. The equivalents, calculated from the two alkali branches are: from 9.0 to 10.5, 1875, and from 10.4 to 11.6, 995. The mean value, which is also that of the straight line cutting across the curve between 10 and 11, is 1430. This agrees with the corresponding equivalent, 1390, of ovalbumin.

Numbers of Acidic and Basic Groups.

The comparison will be made as nearly as possible for those parts of the curves which first become linear on each side of the inflection.

Ovalbumin

$$\frac{\text{Equivalent, } p_H = 3 - 4}{\text{Equivalent, } p_H = 10 - 11} = \frac{4.0}{4.7} = 0.85.$$

Serum albumin

$$\text{Corresponding ratio} = 4.0/4.2 = 0.95.$$

In both cases, on the acid side, the buffering power is greater, the equivalent weight less and the number of reacting groups per 1 gm. of protein is slightly greater. Therefore the groups which come into play on each side of neutrality are nearly equal, but the basic (M) or the acidic (A) are slightly more numerous. The separation into two branches, which was possible on account of the smoothness of the serum albumin curve, gives some information as to the proportions of fission products. The constants which come into consideration are presented as exponents of acid constants, although on theory (A) they are really basic constants.

Histidine.	Histidine.	Arginine.	Lysine.	Arginine.
pK_2	pK_3	pK_2	pK_3	pK_3
6.0	9.0	9.1	10.7	13.2

It is clear that if these are the predominant amino acids which determine the behaviour of albumin on the alkaline side, an inflection is to be expected, the centre of which is at about 7.5, agreeing with experimental values. The absence of inflection at the higher p_H values is also accounted for. The buffering power in the neighbourhood of 9.7, which is controlled by lysine, arginine and histidine, but may be slightly diminished by the beginnings of an inflection due to the distance from the pK 's, is less than that in the neighbourhood of 11.0, due to lysine alone. As p_H approaches 12, the influence of arginine pK_3 will be more prominent. A determination of the buffer value may therefore furnish information as to the relative amounts of arginine present. The maximum buffering power of arginine will be at 13.2 and no inflection will be found until the very high alkalinity of 13.8 is reached, Birch and Harris (1930).

The proportion of arginine therefore accounts for the absence of any inflection on the alkaline sides of the combination curves.*

Such high alkalinities are likely to hydrolyse the proteins and also necessitate a large "blank" correction. By working quickly, with serum albumin, which was treated with the required amount of alkali, and saturated with hydrogen while warming up to the thermostat temperature, we have obtained the following results.

* Recent results have demonstrated that arginine and lysine are among the fission products (Cohn, Hendry and Prentiss, 1925).

Table IV.—Serum Albumin at High Alkalinities.

$x = \text{c.c. } 0.1 \text{ N NaOH to } 1 \text{ gm. albumin} \dots\dots$	17.8	19.4	20.7	21.5	18.6	19.4
$p_H \dots\dots\dots$	11.70	11.84	11.86	11.90	12.0	12.05

These results, obtained in three separate series, give a very flat curve, with the high buffering power, $10^4 B = 11.4$, which corresponds to an apparent equivalent weight of 505, *i.e.*, this weight of albumin contains one equivalent of the strongly basic guanidine group. Since the molecular weight of arginine is 174, the above result indicates that the albumin contains 34.5 per cent. of arginine. This, however, is an upper limit, since the lysine contributes a considerable proportion of the buffering power at $p_H = 11.9$. In addition, some unknown factor must contribute to this high buffering power.

Dilute Solutions of 0.1 per cent. Albumin.—As is seen from Table I above and fig. 1, the p_H values both on the acid and on the alkaline side diminish in a perfectly regular manner on dilution, the buffer values per 1 gm. of albumin increase on dilution.

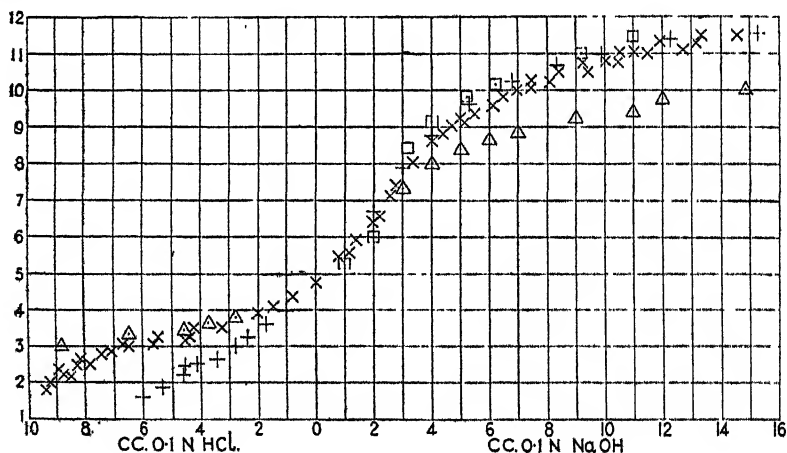


FIG. 1.

The combination of 1.0 and 0.1 per cent. Serum Albumin with acids and alkalis.

\times 1.0 per cent. solution.

Δ 0.1 per cent. solution.

$+$ Heat denatured.

\square Ovalbumin 1 per cent. solution
heat denatured.

The 0.1 per cent. Solutions and Hydrolysis.—The curves of the 0.1 per cent. solutions diverge from those of the 1.0 per cent. in the directions which are to be expected from the increased hydrolysis of the former. From a little below

the isoelectric point at $p_H = 4.5$ to $p_H = 7.2$ the two curves are nearly identical; the buffering power of serum albumin only varies very slightly with the concentration over this range. The slight effect of diluting blood plasma in certain colorimetric tests must be referred therefore to the carbonate buffers rather than to the serum.

If pK_B had been equal to pK_A (M theory) the curves of the 0.1 per cent. solutions would have crossed those of the 1 per cent. solutions symmetrically at the neutral point. But it is known that $pK_B = 10$ ca. and $pK_A = 6$ ca. Hence the crossing point is at pI , and the curves do not diverge symmetrically. For small differences from $p_H = 4.7$, the divergence at first is greater on the acid than on the alkaline side. Thus if Δv is the difference between the c.c. of 0.1 N reagent required to bring p_H to a given value (a) in 0.1 per cent., (b) in 1.0 per cent. solution, then at $p_H = 5.7$ $\Delta v = 0$, while at $p_H = 3.5$ Δv is about 0.4 to 0.5. This want of symmetry is, of course, conditional upon the inequality of pK_A and pK_B . Although the two p_H values considered are equally removed from pI , the more alkaline 5.7 is nearer to the neutral point, and other factors being the same, hydrolysis on dilution diminishes as the p_H of the original solution is nearer to 7.

From inspection of the curve it would appear that hydrolysis is more extensive on the alkaline side. This is true, because the p_H values are much farther removed from $pI = 4.7$ and at the same time do not pass into the regions where a very large excess of the titrating reagent is present. Thus $p_H = 9$ corresponds on the acid side to $p_H = 0.4$, which is far beyond the curve and would require an enormous excess of the strong acid.

The question as to whether the hydrolysis is normal can be decided by applying the standard equations. Since the order of equivalent concentration of 0.1 per cent. protein is a little less than 1×10^{-4} and $(H^+) = \text{say } 1 \times 10^{-9}$ to 1×10^{-10} or $(OH^-) = 1 \times 10^{-5}$ to 1×10^{-4} , the degree of hydrolysis is very great. In solutions having $(H^+) = 1 \times 10^{-8}$ and therefore $(OH^-) = 1 \times 10^{-6}$ we can expect to find that the degree of hydrolysis is normal. If we further assume that M does not alter so that the molar concentration $1/M$ in the 1.0 per cent. is 10 times $0.1/M$ in the 0.1 per cent., and further that the apparent constant pK_A does not alter on dilution.

Then the equation

$$p_H = 7 + \frac{1}{2}pK + \frac{1}{2}\log c$$

is written for the two solutions, and by subtraction

$$p_H(1.0) - p_H(0.1) = \frac{1}{2}\log 10 = 0.5.$$

Actually, at $V = 4$ c.c. of alkali :

$$\Delta p_H = 8.6 - 8.1 = 0.5.$$

At $V = 5$ c.c., however, Δp_H is 0.8, and for further increases of v and p_H , Δp_H becomes 1.0 or more. Special equations which have been formulated to deal with very high degrees of hydrolysis may be applied, but the results are indeterminate because in the correcting factor Kw/KC , it cannot be assumed that either C the equivalent concentration of the protein, or K its apparent acidic dissociation constant, are unaltered by the dilution.

Denaturation by Heat.—According to the results of Hendrix and Wilson (1928), which were quoted in our previous paper (*loc. cit.*), ovalbumin which has been coagulated by heat, for 30 minutes in an autoclave, shows a loss of buffering power from pI to about p_H 7.0 but at higher values this is recovered, and the curve passes into our lower combination curve.

We have tested this matter further by heating the ovalbumin for 15–20 minutes over a briskly boiling water bath and then titrating with alkali as usual, Table V.

The natural p_H is 6 to 6.5 instead of about 5.0 as in natural albumin. After adjustment to pI with acid, the addition of alkali gives a combination curve

Table V.—The Titration of 1 gm. of Ovalbumin or Serum Albumin, which has been previously denatured by heat, with 0.1 N alkali or acid, of which “ x ” c.c. are required to bring 100 c.c. of 1.0 per cent. solution from pI to p_H at 25° C.

Ovalbumin.				Serum albumin.			
Alkali titrations.				Acid.		Alkali.	
x .	p_H .	x .	p_H .	x .	p_H .	x .	p_H .
0	6.55	0	6.1	1.0	4.05	1.0	5.6
2.3 acid to	4.8	1.9 acid to	4.8	1.7 (2.0)	3.6	2.0	6.7
1.0	5.25	2.1	6.4	2.36 (3.0)	3.25	3.0	7.9
2.0	6.0	3.15	8.4	2.75 (4.0)	3.0	4.0	8.7
3.0	7.45	4.2	9.3	3.38 (6.25)	2.65	5.3	9.6
4.0	9.1	5.25	9.8	4.5 (8.75)	2.5	6.8 (7.0)	10.2
5.1	9.86	6.2 (6.3)	10.1	4.62 (12.5)	2.2	8.4 (9.0)	10.65
		9.2 (10.5)	11.0	5.3 (27.5)	1.85	9.9 (11.0)	10.95
		11.0 (14.7)	11.48	6.0 (40.0)	1.6	11.6 (13.3)	11.1
		11.4 (21.0)	11.8			13.3 (16.7)	11.4
						15.3 (20.0)	11.55

which shows some loss of buffering power between 8 and 9. It then passes completely into the *upper* curve of untreated albumin, *i.e.*, that which we have considered to be characteristic of the unhydrolysed and natural albumin.

Serum albumin was coagulated by heat in a similar manner. As is evident from Table V and fig. 1, the combination curve with alkali is practically unaffected, there being only a slight loss of buffering power, which is restored on further treatment with alkali, so that the sample then behaves like perfectly fresh albumin. It is far otherwise with the acid titration; serum albumin which has been coagulated by heat shows a great loss of buffering power towards acid.

It may be inferred from these and the previous results that denaturation by heat affects albumins in the opposite direction to denaturation by alkali hydrolysis.

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The Action of Certain Volatile Substances and Gases on the Growth of Mould Fungi.

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Introduction.

The action of certain volatile substances and gases on the germination and growth of mould fungi is of both theoretical and practical interest.

Little attention has been paid hitherto to the manner in which various substances retard the growth of mould fungi or to the differences in their effects.* It is the aim of this paper to consider comparatively some of the more important ways in which growth may be influenced by the presence of certain growth-retarding substances. Previous investigations have usually dealt with the effect of some single substance and have failed, in consequence, to emphasise those characteristics most useful in comparing the action of one substance with that of another. Reference to these investigations will therefore be left until the final discussion when they can be considered in the light of the present findings.

The practical importance of a knowledge of the influence of volatile substances on the growth of mould fungi lies, in part, in the use to which these either alone, or in combination with low temperatures, may be put in the storage of food products and other materials which suffer deterioration or damage through mould growth. It is probable that it is by control of the composition of the atmosphere that most progress in the storage of foodstuffs is likely to be made.

Methods.

The effects of volatile substances can readily be observed by using air-tight glass jars of simple pattern illustrated in fig. 1.

* A preliminary report on the action of certain volatile substances, including acetaldehyde, ether, chloroform, acetone, on the growth of mould fungi was published by the author in the 'Report of the Food Investigation Board, Department of Scientific and Industrial Research, for 1930,' pp. 48-55. A brief account of the action of hydrogen cyanide, hydrogen sulphide, sulphur dioxide and ammonia is included in the same report for the year 1931, which is now in the press.

The bottom of the jars consists of a plain glass cylinder. The stopper is as wide as the base, has a flat top and is hollowed out evenly on the inside. The stopper can be removed, covered temporarily with the lid of a Petri dish, sterilised in the usual way and filled to a suitable depth with nutrient agar. The agar is inoculated in the centre with a spore suspension of the fungus,

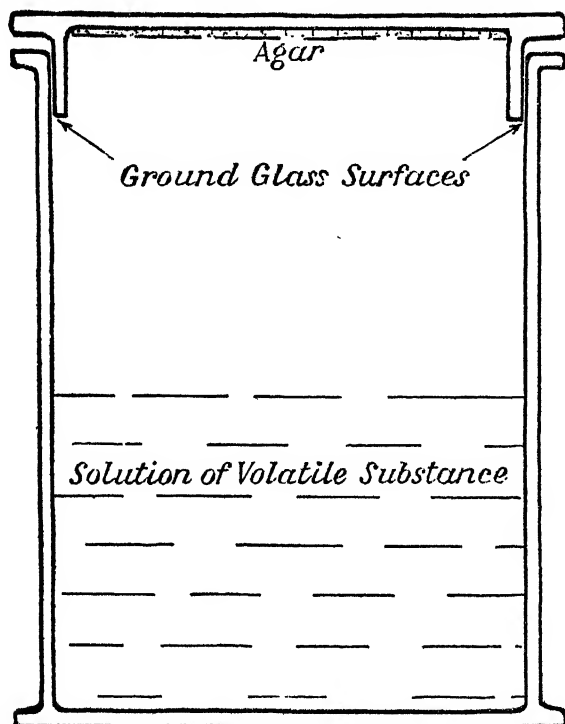


FIG. 1.

the stopper is replaced, and growth takes place across the agar surface exposed within the jar.

Three variables which may affect the rate of growth of fungi can be controlled by the use of jars of this pattern :—

- (1) The humidity of the air can be controlled by enclosing solutions of salts, acids, or bases of known concentrations.
- (2) Definite partial pressures of volatile substances can be maintained by enclosing in the jars solutions of known concentrations of such substances.
- (3) Definite pressures of gases can be maintained in the jar by evolving suitable volumes within the jar. This method of evolving gases within

a closed system is only suitable for use with those gases which affect growth when present in small concentrations.

In the experiments to be described, the jars most often used were about 10 cm. in diameter and 10 cm. in height and of about 800 c.c. capacity. The inside of the stopper was 8-9 cm. in diameter. Jars of this size are probably the most convenient for general use. Smaller reagent bottles possessing the essential characteristic of a hollow stopper have also at times been used for preliminary tests. More frequently it has been necessary to use larger jars some 16 cm. in diameter and $2\frac{1}{2}$ litres capacity.

Care was taken always to follow the same general procedure. A constant amount of agar was used, *i.e.*, 15 c.c. which gave a depth of 2-3 mm. 2 per cent. malt extract agar (p_H 5.8-6.0) was used for the most part, but results with malt agar have been checked by comparison with growth on other synthetic agars. A constant amount of solution of the volatile compound has also been used, though within wide limits the amount of solution used was found not to influence the rate of growth. 400 c.c. of solution were added to the 800 c.c. jars and 1000 c.c. to the larger jars. By using a large bulk of solution appreciable change in concentration of the solution by loss of the volatile compound to the internal atmosphere was avoided. The solutions were constantly renewed, especially when changes in the growth rate were observed. Care was always taken that the solutions were warmed to the correct temperatures before they were poured into the jars.

When gases were to be evolved within the jar 25 or 50 c.c. of $N/2$ sulphuric acid were placed in the bottom of the jar and a suitable amount (*e.g.*, 1, 2, 3 etc., c.c.) of a standard solution of either potassium cyanide, sodium sulphide or sodium sulphite was added in small tubes. Special precautions were taken in preparing standard solutions of sodium sulphide and sodium sulphite, which readily hydrolyse. They were made alkaline to reduce hydrolysis to a minimum and stock solutions were constantly renewed. Acid and salt solutions were only allowed to mix after the stopper had been replaced. In expressing concentrations it has been assumed that all the gas formed by interaction of acid and salt was present in the atmosphere. When the action of a gas was being investigated the jars were opened each day, the gas washed out, and the required concentration again generated. It was found by trial that this was best done after the first 12 hours and subsequently after every 24 hours.

Growth across the agar surface was measured every morning and evening at

approximately 12-hour intervals. This could be done without opening the jars. All measurements recorded were for the diameter alone.

The graphs recorded here illustrate the growth of *Trichoderma lignorum* which has been used in all cases. In addition, the effects of acetaldehyde and other volatile substances and some gases on the growth of other fungi, including *Rhizopus nigricans*, *Thielaviopsis paradoxa*, *Glaeosporium musarum*, and *Botrytis cinerea*, have been observed and have been found to be similar to the effects of these substances on the growth of *Trichoderma lignorum*.

*Growth in Air and in the Presence of Certain Volatile Compounds
and Gases.*

(1) *Growth in Air*.—Brown (1923) has discussed certain aspects of the behaviour of fungi when grown on solid media and the value of size of colony (diameter) as a measure of growth.

Growth on an agar plate is regular in every direction, and the colonies produced are perfectly circular in outline.

Henderson Smith (1923, 1924) observed that the hyphal extension was strictly apical. This is readily seen to be so by observing growth of the margin of a colony under a microscope.

If the size (diameter) of the colony is plotted against time a characteristic curve is obtained. Growth, at first slow, increases in rate to a maximum which may or may not be maintained.

Brown (1923) states: "The general feature of these curves is that in the early stages the rate of growth is small, and that it then rises to a maximum which may or may not be maintained. Fungi which keep up this limiting rate of growth are described as being of the non-staling type, those in which the rate of growth falls off from the maximum are described as being of the staling type." Brown has studied the "staling" type of growth in great detail. It has, however, been my experience that the non-staling type of growth is encountered more commonly than the staling type.

The composition of the medium determines in some measure the rate of spread of the colony but it influences more especially the density and general appearance of the mycelium. Size alone is in consequence not suitable for comparing the effects of different media on growth. The measurement of size seems well suited, however, to the measurement of the effect of some external factor which is found to check the rate of spread without noticeably influencing the density of the mycelium. It cannot, however, be too strongly

emphasised that the exact relation between rate of marginal spread and size of colony has not yet been accurately established.

(2) *Germination and Growth in the Presence of Acetone and Acetaldehyde.*—Fig. 2 records the size of colonies of *Trichoderma lignorum* grown in the presence of acetone. Fig. 3 records the size of colonies of the same fungus grown in the presence of acetaldehyde.

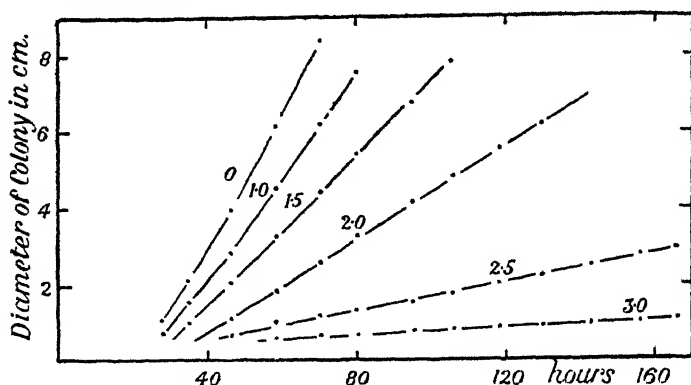


FIG. 2.—Growth of *Trichoderma lignorum* at 25° C. in the presence of acetone solutions of 0, 1.0, 1.5 parts per 100.

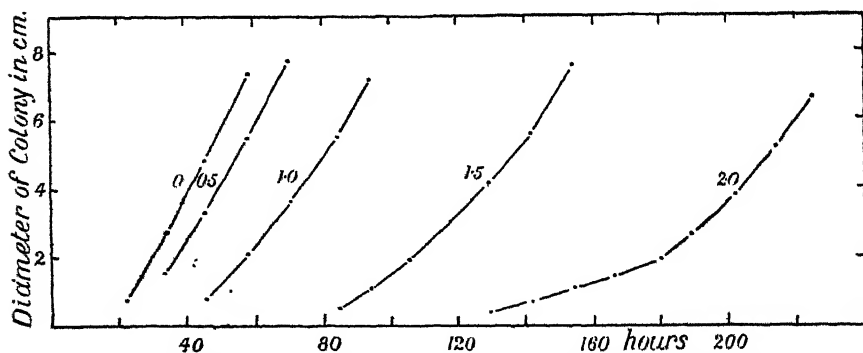


FIG. 3.—Growth of *Trichoderma lignorum* at 25° C. in the presence of acetaldehyde solutions of 0, 0.5, 1.0, 1.5 parts per 10,000.

Certain characteristic differences are obvious when the two graphs are compared.

In the presence of acetone it is observed that :—

- (1) There is very little delay in the time required for growth to be noticeable when compared with the time taken in air.
- (2) The rates of increase in size of colonies at the various concentrations of acetone remain constant.

In the presence of acetaldehyde it is observed that :—

- (1) There is a noticeable delay in the time for growth to become visible when compared with the time taken in air.
- (2) The rates of increase in size of colonies at the various concentrations of aldehyde do not remain constant but increase as the colony becomes larger.

The differences observed in the time taken for growth to be visible is in part due to the differences in the effect of the volatile substances on the time required for germination, i.e., the latent period of germination.

Spores of *Trichoderma lignorum* sown on nutrient agar and exposed to atmospheres containing acetone and acetaldehyde respectively were examined at regular intervals. The times in hours for germ tubes to appear are recorded in Table I. In every instance germination was regular, uniform and practically complete.

Table I.

	Parts of acetone per 100. Parts of acetaldehyde per 10,000.							
	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5
In presence of acetone	13-16	13-16	13-16	13-16	16-23	16-23	23-33	No germ in 140 hours.
In presence of acetaldehyde	13-16	16-23	23-33	43-58	82-106	No germ in 130 hours.		

Whereas in the presence of acetone germination is only slightly delayed even at those concentrations almost as great as those inhibiting germination and growth, in the presence of acetaldehyde germination is delayed somewhat in the lowest concentrations used and still more as the concentration is increased. Such a delay in germination clearly causes a delay in the appearance of the colony and this in part accounts for the differences which are observed in the time for the colony to become visible.

The table clearly shows one of the various ways in which germination may be affected by some external factor and indicates the importance of distinguishing between the effect a substance may have on (a) the latent period of germination, (b) the percentage germination, and (c) the rate of elongation of the germ tubes.

(3) *Growth following the Transfer of Colonies from Air into the Presence of Volatile Compounds.*—Figs. 4 and 5 record the size of colonies growing in air and of colonies which, allowed to grow in air for various times, were then transferred to atmospheres containing known concentrations of acetone and acetaldehyde respectively.

Characteristic differences are again apparent.

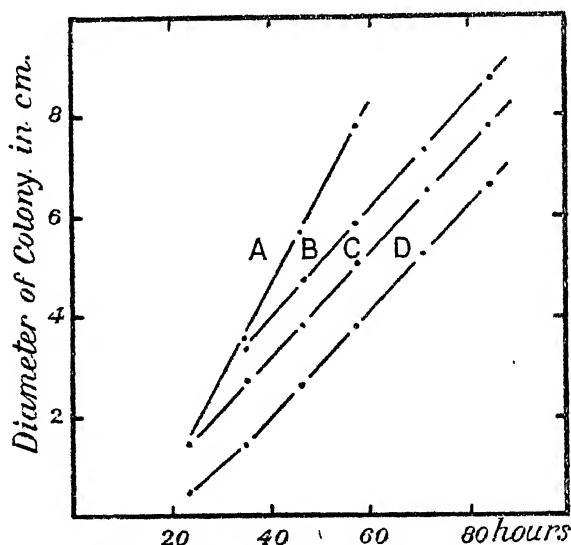


FIG. 4.—Growth of *Trichoderma lignorum* at 25° C. in air (A), and when transferred to the presence of a solution of acetone of $1\frac{1}{2}$ parts per 100 after 35 (B), $23\frac{1}{2}$ (C) and 0 (D) hours' growth in air.

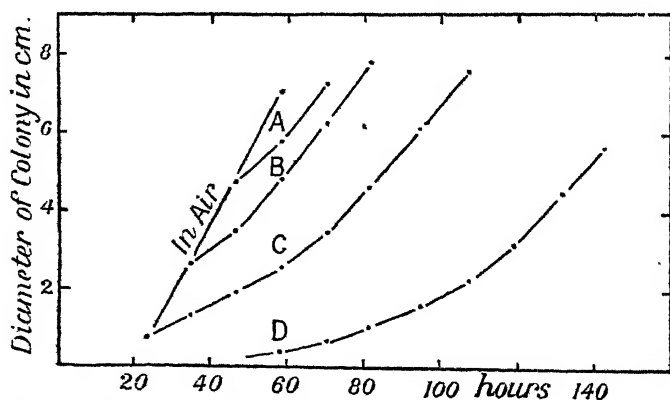


FIG. 5.—Growth of *Trichoderma lignorum* at 25° C. in air and when transferred to the presence of a solution of acetaldehyde of 2 parts per 10,000 after 46 (A), 34 (B), 22 (C) and 12 (D) hours' growth in air.

When transferred from air to an atmosphere containing acetone :—

- (1) The rate of growth is quickly reduced to, and remains constant at a value determined by the concentration.
- (2) The size of the colony before transfer does not influence the subsequent rate of growth. At any concentration the rate of spread is the same whether germination began in air or in the presence of the acetone.

When transferred from air to an atmosphere containing acetaldehyde the changes in the growth rate are much less simple :—

- (1) The rate of growth is immediately decreased. Growth may even be stopped.
- (2) The growth rate does not remain at the initial low value. It increases with time of exposure.
- (3) The extent of the initial reduction of the growth rate, the time of cessation of growth, and the rate of recovery of growth depend on the size of the colony.
- (4) The initial reduction in the growth rate is greater for a small than for a large colony.
- (5) The concentration of acetaldehyde needed to inhibit germination is less than that required to inhibit the growth of a colony. At 25° C. germination is inhibited by concentrations of 2–2½ parts per 10,000. Growth may continue in concentrations of 5 parts per 10,000, provided the colony has already reached some 3 cm. in diameter.
- (6) The increase in the rate of growth does not continue indefinitely but reaches a constant value. In fig. 5 the curves, after first increasing in slope, straighten and become parallel with one another. The value of this constant rate of growth is determined by the concentration of the acetaldehyde. Comparing the final rates of growth at the various concentrations of acetaldehyde it is observed that the extent of reduction is directly proportional to the amount of aldehyde present.

(4) *The Action of other Volatile Compounds.*—The influence of chloroform, ether, ethyl alcohol and amyl formate on the growth of *Trichoderma* has also been observed.

All these substances retard growth in a manner similar to acetone, *i.e.*, in their presence germination is not long delayed, the rate of spread at any concentration remains constant and does not increase with time of exposure, and the concentrations which inhibit germination also inhibit growth.

The strengths of solutions which maintain partial pressures of these substances sufficient to inhibit growth at 25° C. are those greater than

- 5 parts of chloroform per 10,000 ;
- 6 parts of acetal per 1000 ;
- 3½ parts of ether per 1000 ;
- 1½ parts of amyl formate per 1000 ;
- 3 parts ethyl alcohol per 100.

The action of propionaldehyde and butyraldehyde, the two homologues next above acetaldehyde in the aliphatic aldehyde series, also retard germination and growth in the same way as acetaldehyde.

On the other hand, formaldehyde, the first member of the series, retards growth in a manner similar not to that of acetaldehyde but to that of acetone, chloroform, etc. Formalin* solutions of 1, 2, 3 and 4 parts per 1000 give partial pressures of formaldehyde sufficient to retard but not to inhibit growth.

(5) *The Influence of Temperature on Growth in the Presence of Volatile Compounds.*—The relations described above hold quantitatively for all temperatures which have been used.

The concentrations of the volatile substances needed to inhibit growth or germination do vary, however, with the temperature. For example at 25° C. germination is possible in the presence of solutions of acetaldehyde of concentrations up to 2½ parts per 10,000, at 18° only in concentrations up to 1½ parts per 10,000.

At 25° C. growth continues in concentrations of not more than 5 parts per 10,000, at 18° in concentrations of not more than 3 parts per 10,000.

The concentrations of the acetone solution needed to inhibit germination and growth are approximately 3 per cent. at 25° C., 2½ per cent. at 18° C., and 2 per cent. at 15° C.

The influence of temperature on the concentration of an inhibitor needed to prevent growth has been noted before. Brown (1922) states that "the relative retarding effect of a given concentration of carbon dioxide is greater at a low than at a higher concentration." The writer (1929) has also stated that "the further the temperature is removed from the optimum the narrower is the range of humidities allowing germination."

(6) *Germination and Growth in the Presence of certain Gases.*—Figs. 6, 7, 8 and 9 show the size of colonies of *Trichoderma lignorum* grown in the presence of hydrogen cyanide, hydrogen sulphide, sulphur dioxide and ammonia. The

* 40 per cent. solution of formaldehyde.

first three gases were evolved into the atmosphere of the jar by mixing salts with acid. The concentration of ammonia was maintained by introducing into the jar a solution formed by diluting a concentrated ammonia solution,

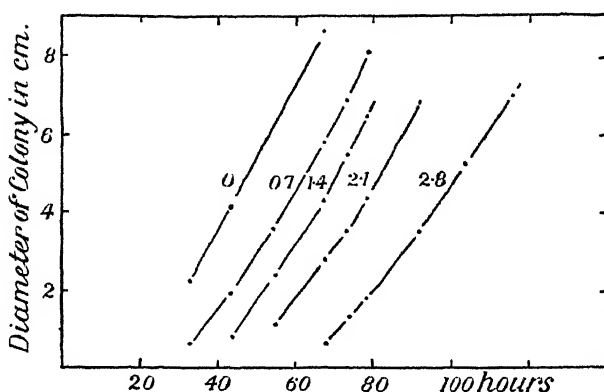


FIG. 6.—Growth of *Trichoderma lignorum* at 25° C. in the presence of hydrogen cyanide of concentrations of 0, 0.7, 1.4, 2.1, 2.8 c.c. per 1000.

sodium hydroxide being added to remove the carbon dioxide formed in respiration.

Germination and growth were observed in concentrations of hydrogen cyanide not greater than 2.8 c.c. per 1000, hydrogen sulphide not greater than

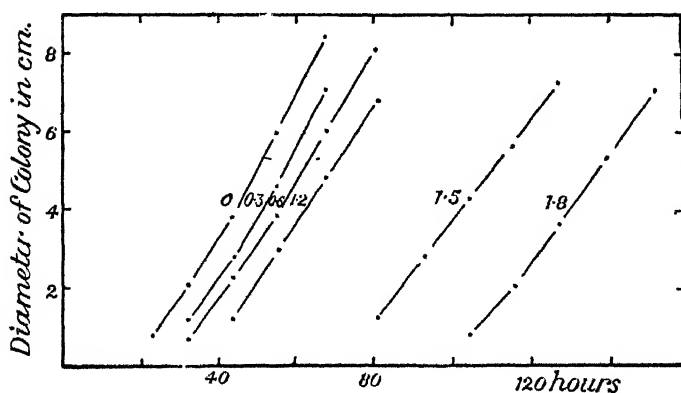


FIG. 7.—Growth of *Trichoderma lignorum* at 25° C. in the presence of hydrogen sulphide of concentrations of 0, 0.3, 0.6, etc., c.c. per 10,000.

0.18 c.c. per 1000, sulphur dioxide not greater than 0.9 c.c. per 1000, and in the presence of a solution of ammonia not stronger than 1 c.c. concentrated ammonia solution per 10,000 c.c.

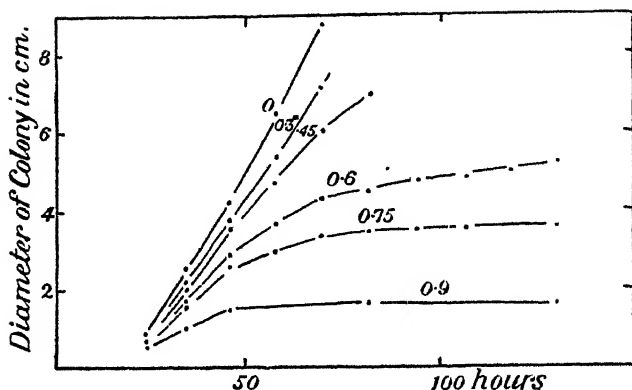


FIG. 8.—Growth of *Trichoderma lignorum* at 25° C. in the presence of sulphur dioxide of concentrations 0, 0.3, 0.6, etc., c.c. per 1000.

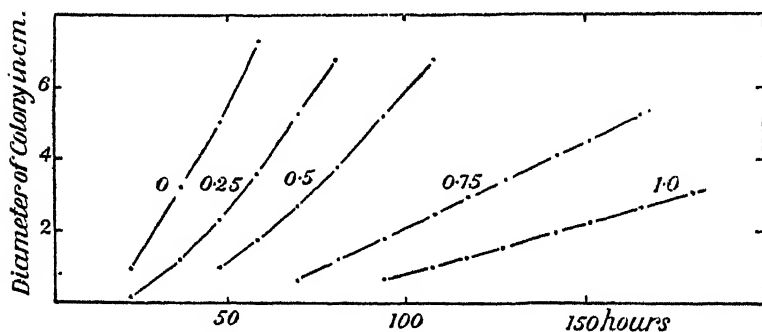


FIG. 9.—Growth of *Trichoderma lignorum* at 25° C. in the presence of ammonia solutions of 0, 0.25, 0.5, etc., c.c. of 0.88 ammonia per 10,000.

In the presence of hydrogen cyanide or hydrogen sulphide the growth of the colony is delayed. The rate of spread is also at first somewhat reduced but rapidly rises to a value approaching that in air.

In the presence of sulphur dioxide the appearance of the colony is not delayed. The rate of spread decreases, however, with the time of exposure and even ceases at the higher concentrations.

In the presence of ammonia the growth of the colony is delayed. The spread of the colony is subsequently regular or increases slightly in rate.

(7) *Growth following the Transfer of Colonies from Air into the Presence of these Gases.*—When hydrogen cyanide or hydrogen sulphide is introduced into the air surrounding a growing colony growth is checked in a characteristic way:—

- (1) There is an immediate cessation of growth or a marked reduction in the rate of spread.

- (2) The extent of the initial reduction depends not only on concentration but also on the size of the colony.
- (3) The growth rate increases with time of exposure. When hydrogen sulphide is used there is first a decrease, then an increase which is followed by another decrease. These changes are considered to be due to the effect of the time taken for the concentration of the hydrogen sulphide in the agar to come into equilibrium with that in the air, superimposed on the normal growth reaction.
- (4) The rate of growth does not increase indefinitely but reaches a constant value. There is one very marked distinction, however, between the action of hydrogen cyanide and that of hydrogen sulphide, viz. :—
 - (a) The final value of the rate of spread attained in the presence of hydrogen cyanide is not altered by varying the concentration of the gas from 5 to 16 c.c. per 1000. Fig. 10 illustrates this and also the varying periods during which growth is inhibited after the introduction of the gas at various concentrations.

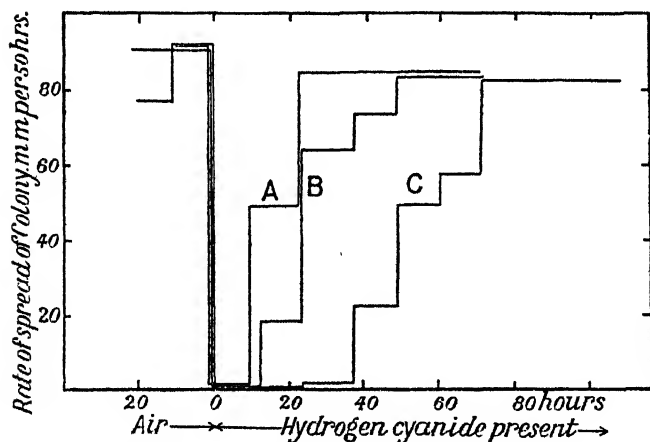


FIG. 10.—Rate of growth (rate of increase of diameter in millimetres per 50 hours) of *Trichoderma lignorum* at 25° C. when transferred from air to the presence of hydrogen cyanide of concentrations of (A) 6.8, (B) 11.25, and (C) 15.75 c.c. per 1000.

- (b) The final value of the rate of spread attained in the presence of hydrogen sulphide is determined by the concentration of the gas, the reduction in the rate being directly proportional to the amount of gas present, fig. 11.
- (5) The concentrations of hydrogen cyanide and hydrogen sulphide needed to inhibit growth is much in excess of that needed to inhibit germination.

For example germination is checked by 0.2 parts of hydrogen sulphide per 1000, while growth continues in concentrations of 0.6 parts per 1000. Even in concentrations of 0.6–1.2 parts per 1000 there is an increase in the rate of spread after an initial reduction, but growth finally ceases.

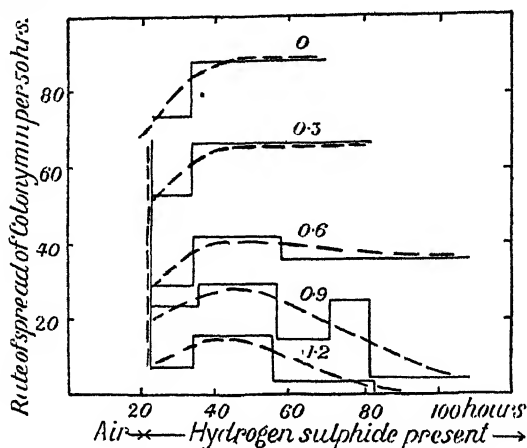


FIG. 11.—Rate of growth (rate of increase of diameter in millimetres per 50 hours) of *Trichoderma lignorum* at 25° C. when transferred from air to the presence of hydrogen sulphide of concentrations 0, 0.3, 0.6, 0.9 parts per 1000.

When sulphur dioxide or ammonia is introduced into the atmosphere surrounding growing colonies the rate of spread is retarded :—

- (1) The rate of growth is slowly reduced till a final constant value is reached.
- (2) The changes in the rate of spread are not influenced by the size of the colony and are determined solely by the concentrations of the gases.
- (3) The concentrations of sulphur dioxide and ammonia needed to inhibit growth are the same as those needed to inhibit germination.

Comparison of the Action of Volatile Compounds and certain Gases on the Rate of Growth.

The action of hydrogen cyanide and hydrogen sulphide on germination and growth is similar to that of acetaldehyde in the following respects :—

- (1) The latent period of germination is increased.
- (2) The growth rates following germination are much reduced but they increase as the colony becomes larger.
- (3) The concentration required to inhibit growth is greater than that required to check germination.

- (4) When colonies are transferred from air into the presence of these substances the growth rates are at first reduced and then increase to a constant value.

Sulphur dioxide retards growth in a manner which in some respects resembles the action of acetone :—

- (1) Germination is not delayed beyond germination in air.
- (2) The growth rate does not increase as the colony becomes larger. On the contrary the rate of spread at first decreases. The decreasing rate of spread with time of exposure to a given concentration is believed to be due to the slowness with which the sulphur dioxide in the agar comes into equilibrium with that in the air—a view supported by the differences in the rate of falling off which are observed when the gas is renewed at varying intervals of time.

The action of ammonia on the other hand is quite distinct from that of the other substances which have been studied in that :—

- (1) The latent period of germination is very much prolonged.
- (2) The same concentration is needed to inhibit germination as to inhibit growth.

Discussion.

Reference may now be made to former investigations. Lesage (1895 and 1896) showed that the vapours of certain volatile compounds would prevent germination and also that weak solutions of hydrochloric acid, acetic acid, and alcohol would delay germination somewhat. Latham (1905) weighed the mycelium produced in a culture solution in the presence of small amounts of chloroform. Increases in dry weight were found in the presence of small amounts of chloroform. But only one dry weight estimation was made and it is not possible to judge how the amount of mycelium in the culture was increasing or what his comparative figures really represent. Also the explanation based on the "economic coefficient" as a measure of economy of metabolism neglects so many factors that it cannot be considered seriously.

Reynolds (1924) grew *Fusarium lini* on an agar medium to which potassium cyanide had been added, observed that the early stages of growth were retarded much more than the later stages, and concluded that "the rate in all potassium cyanide concentrations was greater after the first slow period of growth than in cultures free from cyanide." Actually, if the rates of growth are calculated

from his data it is seen that potassium cyanide at first retards growth but that as the colony increases in size it fails to check the rate of spread.

Marsh (1929) measured the germination of spores of *Monilia cinerea*, *Monilia fructigena*, and *Botrytis cinerea* in the presence of hydrogen sulphide but never continued his observations for more than 18 hours. Closer examination shows that higher values are recorded for the percentage germination when the observations were continued longest.

McCallan and Wilcoxon (1931) also measured germination in the presence of hydrogen sulphide but "in all cases the spores were examined for germination after 22 hours." This cannot be considered an adequate period of time for observing the effect of hydrogen sulphide. Moreover there is a failure to distinguish between the percentage germination, the latent period of germination and the elongation of the germ tubes.

It is clear that in most of these other investigations information on those particulars most useful in making comparisons of the effects of these inhibitors is lacking. It is nevertheless clear from the present investigations that volatile substances and certain gases retard the growth of fungi in characteristically different ways.

So far no adequate explanation can be offered wholly to account for these differences, but neither has the action of such substances on other physiological processes been satisfactorily explained. The fact that the actions of hydrogen cyanide, hydrogen sulphide and aldehyde on growth have many characteristics in common which are quite distinct from those associated with the action of chloroform, acetone, etc., is of great interest and offers scope for further investigations.

Hydrogen cyanide, hydrogen sulphide and aldehydes are known to attack the respiratory mechanism of a number of organisms. Chloroform inhibits growth without noticeably affecting respiration (Warburg, 1910), whereas cell division in the presence of cyanides ceases only when respiration has been reduced to a third of the normal value (Loeb and Wasteneys, 1913). Differences in the action of these groups of cell poisons have been observed in several instances. Gray (1924) has noted differences in the action of these substances on ciliary movement, Pantin (1929) on protozoan movement, Warburg (1928) on respiration, Keilin (1929) on the action of various respiratory enzymes, and attempts to explain these differences have led to a better understanding of these processes. They do not, however, help much in explaining the characteristic differences which are observed in the action of the various substances on the growth of mould fungi used in the present investigations.

My thanks are due to Mr. F. T. Brooks, F.R.S., and Dr. F. Kidd, for their help and criticism.

Summary.

Volatile substances and gases which in certain concentrations inhibit germination of the spores of mould fungi may, when present in smaller amounts, retard germination and growth in characteristically different ways.

In the presence of such substances as acetone, chloroform, ether, etc. :—

- (1) The latent period of germination is practically the same as in air.
- (2) The rate of spread of the colony, though reduced, remains constant as the colony increases in size.

The concentrations of these substances needed to inhibit growth are the same as those needed to check germination.

In the presence of such substances as acetaldehyde, hydrogen cyanide and hydrogen sulphide :—

- (1) The latent period of germination is increased considerably beyond that in air.
- (2) The rate of spread of the colony following germination is at first much reduced but increases as the colony becomes larger.
- (3) The rate of spread of a colony when transferred from air to the presence of these substances is at first reduced but increases to a constant value determined by the concentration of the inhibitor.

The concentrations of these substances needed to inhibit growth are greater than those required to inhibit germination.

In the presence of other substances such as ammonia the latent period of germination is increased and germination is possible in all concentrations which do not inhibit growth.

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The Distribution of the Spinal Terminals (Boutons) of the Pyramidal Tract, Determined by Experimental Degeneration.

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(Communicated by Sir Charles Sherrington, F.R.S.—Received May 31, 1932.)

[PLATE 10.]

I.—Introduction.

In a previous paper in these 'Proceedings' (E. C. Hoff, 1932), a report was given of investigations concerning the *boutons terminaux* which constitute the terminals of nerve fibres around dendrites and perikarya in the central nervous system. It was shown that 24 to 48 hours after section of the afferent roots of the spinal cord of the cat, the boutons undergo degenerative swelling and granulation. This degenerative process reaches a climax in 3 days and ends in the complete destruction of the terminals after 4 to 6 days.*

In the present studies, the normal synapses of the monkey have been investigated; further, the phenomenon of degeneration of boutons following section of nerve fibres has been applied as a method for determining the endings of the pyramidal tracts in the cat and the monkey.

* Since that paper was written, Sereni and Young (1932) have demonstrated small nerve terminals resembling mammalian *boutons terminaux* in cephalopods. After section of the mantle connective nerve in these invertebrates, the small terminals become swollen, take the silver stain more deeply, and finally disappear. The degeneration does not extend into the cells which constitute the next link in the nervous chain, and these authors therefore conclude that the nervous system of cephalopods, like that of the vertebrates, is built up of discontinuous neurones. As in the vertebrates also, interconnection between neurones is made by small, specialised terminals.

II.—*Technique.*

Cajal's silver stain [formula (6A), Bolles Lee (1928)], after injection of the fixative *in vivo*, was used in all the animals. It was found that the technique previously described for the cat cords was successful in the monkey, the only modification necessary being the injection of larger quantities of 10 per cent. chloral hydrate fixative than in the cat.

III.—*The Normal Synapse of the Monkey (Macacus rhesus).*

Boutons terminaux and *boutons de passage* occur around dendrites and perikarya in the central nervous system of the monkey as in the cat. Fig. 4 and fig. 6, Plate 10, show them in the form of tiny loops at the end of nerve fibres. Also, as in the cat, the *boutons de passage* occur along the course of nerve fibres. The loops in the monkey are more circular than those of the cat and are coarser, somewhat thicker, and more darkly staining. There are scarcely any of the small internal fibrils sometimes seen in the bouton of the cat. As with the cat, so likewise with the monkey, it is not possible to demonstrate any protoplasmic continuity between the terminal and the neurofibrils of the dendrite or perikaryon to which the bouton is attached.

The boutons of the monkey cord show variations in size and density comparable with those of the cat; in Clarke's column, the largest boutons are $3\ \mu$ by $2.3\ \mu$, the average ones, about $2.5\ \mu$ by $2.5\ \mu$, and the smallest ones, $1\ \mu$ by $1\ \mu$ or even less. The cells here are small, and on each perikaryon there are from 20 to 50 terminals. Thus, there were 32 boutons on a cell-body $15\ \mu$ by $45\ \mu$, and 30 on one $30\ \mu$ by $14\ \mu$. The boutons of the dorsal horn are slightly larger, the average size being about $3\ \mu$ by $1.6\ \mu$. There are many terminals measuring about $1\ \mu$ by $1.5\ \mu$, also a number of very minute, delicate loops much less than $1\ \mu$ in length. Farther ventrally in the grey matter, the average size of the boutons becomes somewhat less than in the dorsal horn; for instance, on the cells of the ventro-mesial group, the boutons were from $2.3\ \mu$ by $1.5\ \mu$ to $1.4\ \mu$ by $1\ \mu$. On the cells of all groups of the ventral horn the minute loops under $1\ \mu$ in size were very common. The average density of the boutons in the ventral horn was about one terminal per $18\ \mu^2$ of cell- or dendrite-surface. In general, there were fewer terminals per μ^2 in the monkey than in the cat. The monkey upon which these observations were made was only about 18 months old; it is probable, therefore, that it had not yet acquired its full number of synapses.

Boutons occurred not only within the grey matter proper, but, as was the case in cats, they were found also applied to thick fibres in the projections of the grey matter into the ventral columns of white matter. Here, the terminals end presumably around dendrites whose cell bodies lie within the grey matter proper, but no actual case of a dendrite proceeding from a cell into one of these processes, such as was seen in the cat, could be found.

IV.—*The Termination in the Spinal Cord of Pyramidal Tract Fibres.*

A.—*Previous Investigations.*—Using the Marchi method, early workers were able to trace degenerating fibres from the pyramidal tract into the grey matter: Sharpey-Schafer (1899) demonstrated that after experimental lesions involving cortico-spinal fibres of cats and monkeys, degenerated nerve fibres could be seen passing from the pyramidal tract into the grey matter toward Clarke's column. Here they curved around the ventral aspect of that column and lost themselves as fine fibres amongst Clarke's cells. He was unable to trace any fibres from the pyramidal tract into the ventral horn or into any part of the grey matter other than the base of the dorsal horn and Clarke's column.

Collier and Buzzard (1903) using the Marchi method, studied cords of human patients who had died of transverse lesions of the cord at various levels. From 16 cases examined by this method, they found it possible, in two instances, to demonstrate the terminations of the pyramidal tract. At the level of the tenth, eleventh and especially twelfth thoracic segments, they observed degenerated fibres running from the pyramidal tract directly to the front of Clarke's nucleus, breaking up into fine branches among its cells. They also found it impossible to trace fibres into any part of the ventral horn.

Collier and Buzzard called attention to a source of error in the use of Marchi's stain, in that the distal portions of long, degenerated neurones are apt not to stain by this procedure. However, Sherrington [(1901) addendum to Grünbaum and Sherrington] showed that in the chimpanzee, lesions of the hand area in the motor cortex resulted in a degeneration of fibre-filaments traceable into the ventral horn of the grey matter of the crossed side, especially in the lowest brachial segments. Later (1917), Leyton (Grünbaum) and Sherrington described these fine degenerated fibres in the ventral horn following various experimental lesions in the motor cortex of the opposite side. By the use of Sharpey-Schafer's combination of the methods of Marchi and of Kulschitzky, they observed a "peppering" of the whole of the cross-area of the ventral horn of the grey matter with minute degenerated fibres appearing as small dark bodies

without axis cylinders, and easily distinguishable from the normal fibres which stained as minute blue-black rings surrounding a pale axis cylinder.

Using the Marchi-Kulschitzky method, it thus became possible to see extremely small degenerated fibres in the grey matter. However, with the aid of Cajal's silver stain, the degeneration of a fibre can be followed further still, in fact, to the actual terminal. The present investigation, therefore, was undertaken to make a more precise determination by this method of the pyramidal tract endings in the cat and rhesus monkey.

B.—*The Terminals of the Pyramidal Tract in the Spinal Cord of the Cat.*—In a series of seven cats, the cerebral cortex was aseptically exposed under dial anaesthesia, and the gyrus cruciatus with a certain amount of the surrounding cortical tissue was completely removed. In each case, the cat recovered and was killed 72 hours after the operation by injection of 10 per cent. chloral hydrate under ether anaesthesia. On examination of Cajal preparations of these cords, it was found that the grey matter of the crossed side was peppered with degenerated terminals similar to those previously seen in de-afferented cats killed 3 days after operation. These terminals were enlarged, swollen and granular, some being greatly elongated while others were nearly circular. An example of a degenerated bouton is illustrated in fig. 7, Plate 10.

Degenerated terminals occurred in the dorsal as well as in the ventral horn and on cell-bodies and their dendrites. Their distribution is shown in the series of drawings of the cord at various levels in fig. 1, and fig. 2. (The round black dots indicate the location of perikarya upon which degenerated boutons were found, the triangles indicate dendrites with such terminals, and the crosses, peridendritic degenerated terminals occurring very close to the cell-bodies.) In C1, the degenerated endings are quite evenly distributed over the whole area of the grey matter of the crossed side, and in the ventral horn at this level, there is a preponderance of peridendritic endings. The ventro-mesial group on this side shows very few degenerated boutons, while the uncrossed side is also very sparsely supplied.

In C2, the degenerated terminals are seen principally on cell-bodies. On the crossed side, they occur in the ventral horn, and also in the dorsal horn, particularly on cells in the group indicated in fig. 1, by X. It is around these cells that degeneration of boutons is especially noticeable after de-afferentation. There are more degenerated boutons on the uncrossed side in this segment than in C1.

C3 and C4 shows the greatest density of degenerated terminals on the crossed sides, as well as the most scattered distribution, but the number on the

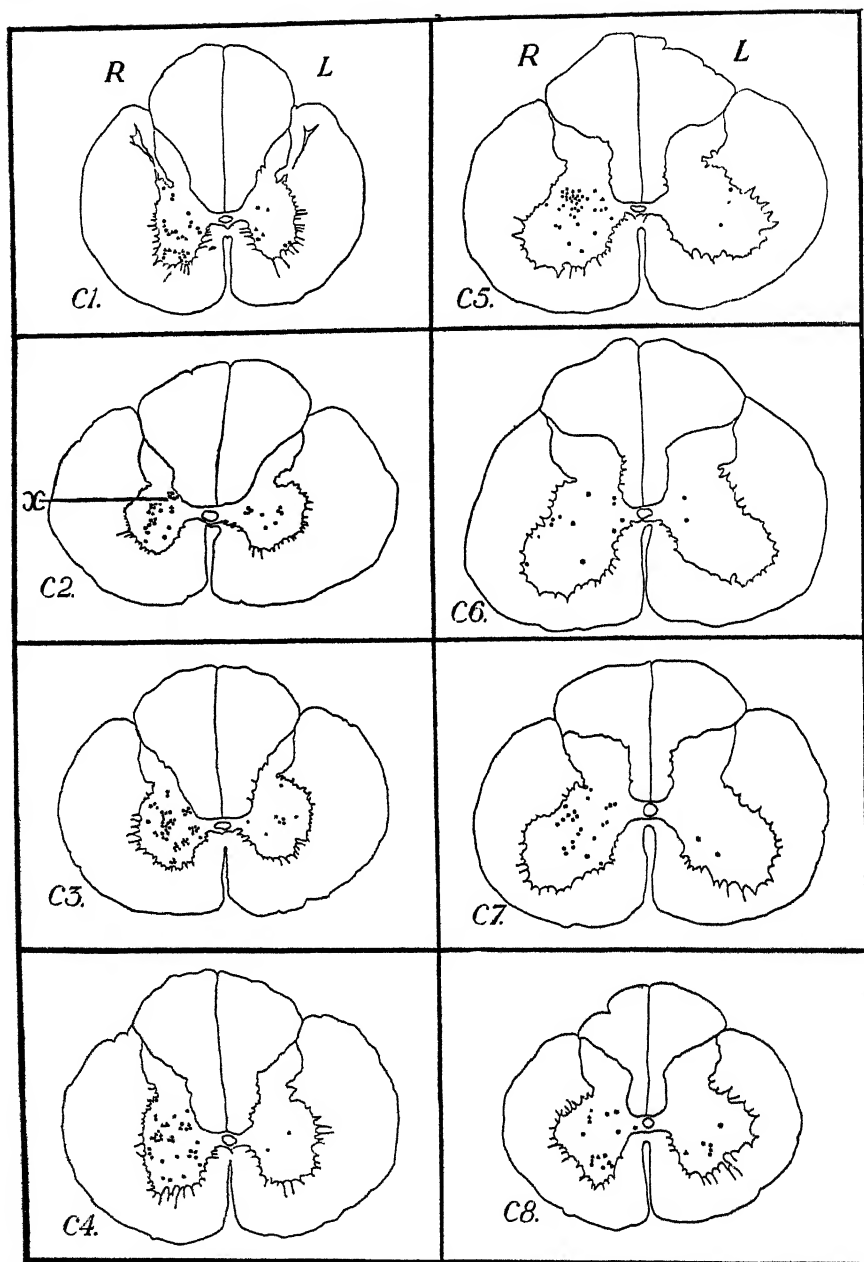


FIG. 1.—Drawings of sections from the first to the eighth cervical segments of a cat, which had been killed 3 days after destruction of the left motor area of the cortex. The solid black circles indicate the position of cell-bodies upon which degenerated boutons were seen, the triangles indicate dendrites showing such terminals, and the crosses represent peridendritic degenerated boutons occurring close to the cell-body. Cat 29. Operation 36. (Camera lucida drawings.)

uncrossed side begins to decrease again. In C5 the greatest density is in the mid-region of the grey matter on the crossed side—the area in which the afferent roots have their terminals. In C6, C7 and C8, the ventro-lateral groups of cells are almost free of degenerated endings and the uncrossed side also contains very few. The density on the crossed side diminishes in C6 but increases again in C7.

In the thoracic region, there are only occasional degenerated boutons and these occur in the ventral horn. No boutons undergoing degeneration were found in Clarke's column, although normal terminals around cells of the column were well stained.

In the post-thoracic segments, degenerated terminals again appear. They are not so dense in this region as in the cervical enlargement, but on the side contralateral to the cerebral lesion they are as widely scattered. On the uncrossed side, however, degenerated endings are seldom observed. The drawings in fig. 2 illustrate the distribution of the pyramidal terminals in post-thoracic segments at various levels.

C.—*The Terminals of the Pyramidal Tract in the Spinal Cord of the Monkey.*—In a young monkey about 18 months old, the arm area of the cortex on the right side was destroyed with full asepsis. The animal recovered very rapidly, and was killed 72 hours after the operation by injection, under ether anaesthesia, of 500 c.c. of 10 per cent. chloral hydrate. Cajal preparations of this cord showed large numbers of degenerating boutons similar to those occurring in the cat. Fig. 5, Plate 10, shows the elongated, granular type of degenerated terminal, while fig. 8 illustrates the swollen, circular type which is of common occurrence. Surrounding this bouton, a few normal ones can be seen.

The distribution of degenerated endings in the monkey is illustrated in the series of drawings shown in fig. 3. As is indicated here, none are found on the crossed side above the second cervical segment. At C2, however, they are distributed evenly over the entire area of the grey matter on the side opposite the lesion occurring chiefly on dendrites. There is an area of greater density in the dorso-lateral region of the grey matter (indicated in fig. 3, by Y); and in C3, almost all of the degenerated boutons were seen on dendrites in this area where they are very dense. In the whole length of the monkey cord, there are hardly any degenerating terminals on the uncrossed side.

In C4, the degenerated boutons are still largely confined to the dorsal horn, with a few terminals in the mid-region and ventral horn. In C5, C6 and C7, however, the distribution of boutons becomes more extensive and they can be seen peppering almost the entire area of the grey matter on the crossed side.

In these segments, the degenerated terminals are principally peridendritic, and most dense in the dorsal horn. In all segments of the cord, the ventro-mesial group of cells shows very few degenerated endings.

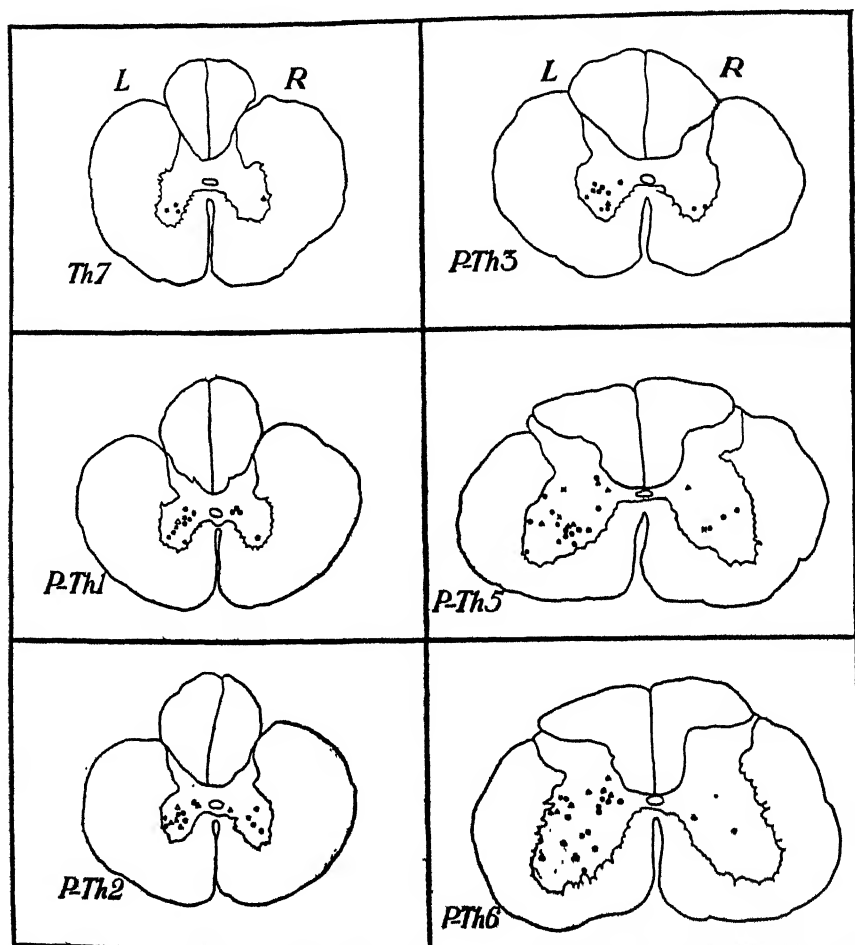


FIG. 2.—Drawings illustrating the terminations of the pyramidal tract fibres in the dorsal and post-thoracic regions of the cord of the cat. The right motor area of the cortex was destroyed 3 days before killing the animal. The circles, triangles, and crosses have the same significance as in fig. 1. Cat 38. Operation 45. P-Th signifies post-thoracic. (Camera lucida drawings.)

Below the seventh cervical segment, they are very sparse on either side of the cord, while in the thoracic region, none are found. Only occasional degenerated terminals are seen in the post-thoracic segments and these occur chiefly in the upper part of the lumbar enlargement. However, one or two

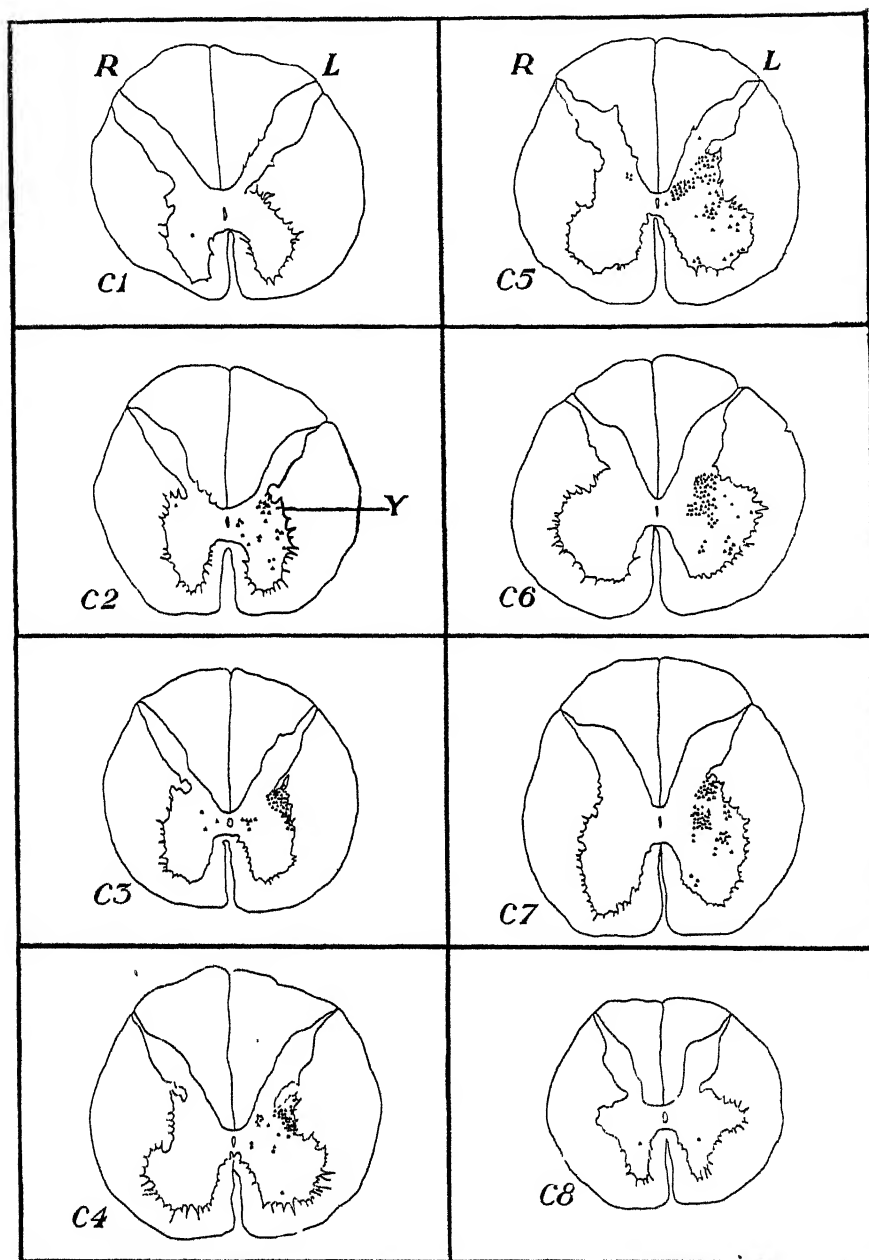


FIG. 3.—Drawings of the sections of the monkey's cord in the cervical region showing the endings of the pyramidal tract fibres in the grey matter. The monkey was killed 3 days after destruction of the arm area of the cortex on the left side. Monkey I. Operation 42b. (Camera lucida drawings.)

degenerated boutons were observed as far down as the seventh post-thoracic segment on the crossed side.

As in the cat, there were no degenerated boutons on cells in Clarke's column.

V.—Discussion.

In agreement with the previous observations of Sherrington, who discovered that in the chimpanzee the collaterals of fibres from the pyramidal tract ramify amongst cells of the ventral horn, the present investigations show that in the rhesus monkey and the cat these fibres actually terminate by means of synapses upon cells of this region. It is further demonstrated that some of the fibres end in the dorsal horn on perikarya and dendrites, including those upon which the terminals of afferent roots of spinal nerves occur, according to previous observations (Hoff, 1932).

This fact that collaterals from the pyramidal tract terminate directly around ventral horn cells offers a histological explanation of Cooper and Denny-Brown's observations (1926, (1) and (2)) on the response in muscles of the fore-limb to cortical stimulation. They found that in monkeys (*Macacus rhesus*, *Cercopithecus mona*, and *Macacus sinicus*) the spinal discharge will follow rhythmic stimulation of the cortex at rates as high as about 180 per second. The transmission of such high rates of stimulation from the motor cortex to the responding muscle, together with the small amount of after-discharge, and the prominence of the primary waves in the electromyogram, suggested to these authors a much more direct connection between the excitable cortex and the spinal motoneurone than might have been expected. The present results support this suggestion, but indicate as well that there is also a considerable termination around neurones of the dorsal horn.

In the cat, no terminals of pyramidal tract fibres were found to be distributed to cells of Clarke's column. In the monkey, the cortical lesion, being confined to the arm area, may not have been extensive enough to involve those cortical cells whose axones end in Clarke's column, and this may account for the fact that no degenerated boutons were found in this region. However, these observations do show that at least no fibres from the arm area of the cortex in the monkey end here.

The number of pyramidal tract fibre endings in the monkey was found to be definitely greater than in the cat; and further in the former animal they are more densely arranged in the grey matter. There also seem to be both relatively and absolutely fewer endings on the side of the cortical lesion in the

monkey than in the cat. In both animals, there are very few terminals in the thoracic region of the cord.

It is possible from these studies to make a rough estimate of the total number of terminals of pyramidal tract fibres in the entire cord. In the case of the cat, the regions from the first to the eighth cervical and the first to eighth post-thoracic segments, a combined length of about 18 cm., there were approximately 24,200 perikarya and dendrites in the crossed side upon which degenerated boutons were seen, and about 4500 in the uncrossed side. There are, therefore, at least 40,000 boutons in the crossed side and 7500 in the uncrossed side which are the terminals of pyramidal tract fibres in the cat. In seven cervical segments of the monkey's cord there are about 16,600 dendrites or cells in the crossed side upon which terminals of fibres from the cortex occurred, and about 400 in the uncrossed side. This means that, in the cord, there are about 30,000 boutons in the crossed side and only about 800 in the uncrossed side which are terminals of fibres from the arm area of the cortex.

These estimates were made on the basis of counts of the total number of perikarya or dendrites, showing degenerated boutons in 12 sections, each 15 μ thick in every segment examined. Thus, in the cat, there were counted in 156 sections in C1 to 8, Th. 7 and P-Th., 1, 2, 3, 5 and 6, 314 such cell-bodies or processes in the crossed side and 58 in the uncrossed side. The total estimate was made by assuming that this was an average for the whole of the cervical and lumbar regions. In the monkey, the same counts were made, there being 387 cell-bodies or dendrites showing degenerated terminals in the crossed side and 10 in the uncrossed side in 84 sections, each 15 μ thick, from C2 to C8. Attention is called to the fact that the figures given are rough approximations only, and are probably too low in that some of the degenerated terminals may have been overlooked.

The author wishes to thank Professor Sir Charles Sherrington for performing, with the author's assistance, the operation on the monkey. It is a pleasure also to express the author's thanks to Dr. H. M. Carleton and Dr. J. C. Eccles for their advice and helpful criticism, and to Mr. H. E. Hoff for assistance in the operations on the cats. The micro-photographs illustrating this paper were made with the camera in the Department of Zoology, Oxford, which Professor E. S. Goodrich very kindly permitted the author to use. The cost of photographic material was defrayed by a grant from the Christopher Welch Trustees.

VI.—*Summary.*

(1) The fact that *boutons terminaux* in the spinal cord degenerate following section of nerve fibres, has been used as a method for determining the endings of the pyramidal tract fibres in the cat and the monkey.

(2) The normal synapse in the monkey resembles that of the cat in consisting of small loop-like *boutons terminaux* and *boutons de passage* occurring around perikarya and dendrites of the cord.

(3) The cats killed 3 days after removal of the motor cortex of the cerebral hemisphere show degenerated boutons in the cord. These occur on the crossed side on practically all cell groups except the ventro-mesial group and Clarke's column. On the uncrossed side the degenerated boutons are found as widely scattered but are less numerous. These degenerated terminals occur in all of the cervical and post-thoracic segments in large numbers, but only a few were seen in the thoracic region.

(4) In a monkey in which the major portion of the arm area of the motor cortex of one hemisphere was destroyed, there were found, in the second to the eighth cervical segments, degenerated boutons similar to those of the cat. On the crossed side they were thickly distributed on both the dorsal and ventral horns, but occurred very sparsely on the uncrossed side.

(5) These studies are in agreement with Sherrington's discovery that fibres from the pyramidal tract ramify around ventral horn cells in the cord of the chimpanzee. They show that in the cat and the monkey, the pyramidal tract fibres actually terminate by means of boutons around ventral horn cells. This fact confirms histologically the suggestion of Cooper and Denny-Brown that the connection between the motor cortex and the spinal motoneurone is very direct, probably involving only one synapse. In addition to the terminals found on the ventral horn cells, many others were seen on cells of the dorsal horn.

(6) On the basis of these investigations, it is roughly estimated that the number of boutons in the cat's cord which are terminals of pyramidal tract fibres is about 40,000 in the crossed side, and 7500 in the uncrossed side; in the monkey, the fibres from the arm area of the motor cortex terminate by means of approximately 30,000 boutons in the side opposite the lesion and only about 800 in the uncrossed side.

PLATE 10.

(In all the figures, the position of the boutons may be found by drawing perpendiculars from the letters indicated.)

FIG. 4.—A normal terminal bouton on a funicular cell of a monkey's cord. (The position of the bouton is indicated by *aa'*.) Third cervical segment. Left side. *Macacus rhesus*, 1. Magnification, 878 diameters.

FIG. 5.—A cell from the left side of the cord of a monkey in which the arm-area of the right motor cortex had been destroyed 3 days before the animal was killed. The bouton, which is seen at *aa'*, is enlarged and granular, and greatly elongated. The intra-



Fig 4.

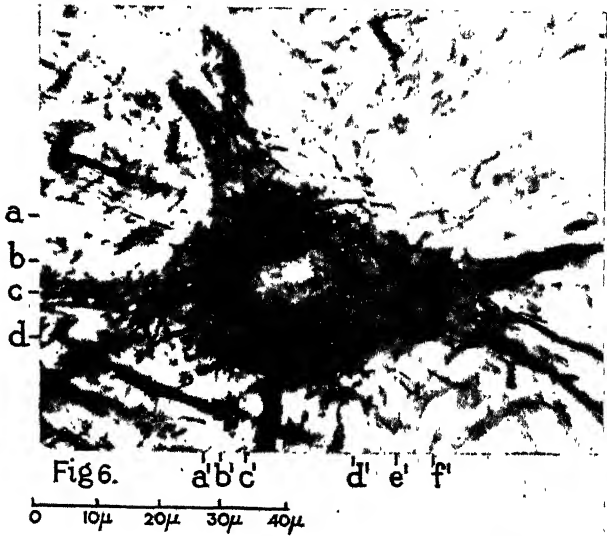


Fig 6.



Fig 5.

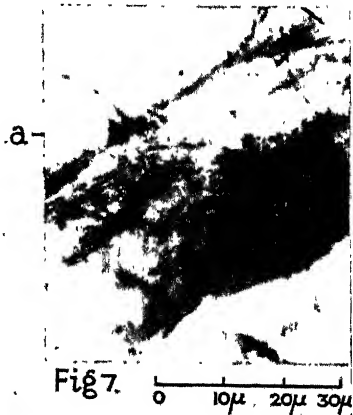


Fig 7.

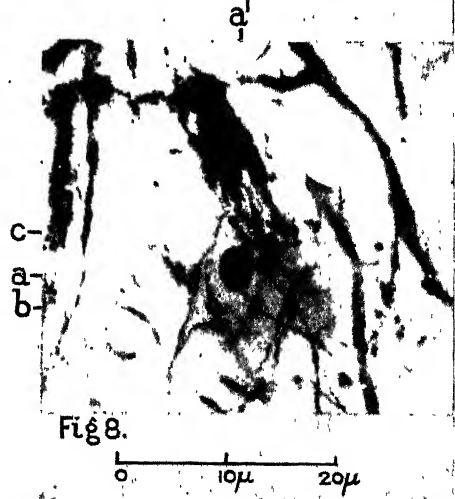


Fig 8.

cellular neurofibrils are unaltered. *M. rhesus*, 1. Operation 42b. Magnification, 1500 diameters.

FIG. 6.—Normal boutons on a cell-body from the spinal cord of the monkey. Third cervical segment. Right side. Attention is called to terminals at *ad'*, *be'*, *cf'*, *ac'* and *db'*. *M. rhesus*, 1. Operation 42b. Magnification, 878 diameters.

FIG. 7.—A terminal bouton, the position of which is indicated by *aa'*, occurring in a cell on the right side of the cord of a cat killed 3 days after extirpation of the motor cortex of the left cerebral hemisphere. From the cervical enlargement. Cat 29. Operation 36. Magnification, 878 diameters.

FIG. 8.—A large circular degenerated bouton, at *aa'*, surrounded by normal terminals, two of which can be seen at *ca'* and *ba'*. This cell occurs on the left side of the cord of monkey 1. Operation 42b. Magnification 1500 diameters.

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*Mesoblastic Tumours following Intraperitoneal Injections of
1 : 2 : 5 : 6-Dibenzanthracene in a Fatty Medium.*

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(From the Research Institute of The Cancer Hospital (Free), London.)

[PLATES 11-13.]

Introduction.

Attention was first drawn to 1 : 2 : 5 : 6-dibenzanthracene as a carcinogenic substance during an inquiry into the fluorescent spectra of coal tars carried out by W. V. Mayneord in collaboration with E. L. Kennaway at this Institute in 1927 and later continued by I. Hieger (1930). Since then the substance has been prepared in a very pure form by J. W. Cook (1931, *a*), who has made some closely allied compounds which are also carcinogenic (1931, *b*, *c*; 1932). Kennaway (1930) and Hieger (1930) have already recorded the causation of epitheliomata by 1 : 2 : 5 : 6-dibenzanthracene dissolved in benzene applied to the skin of mice; and Burrows, Hieger and Kennaway (1932) have described the production of connective tissue tumours in rats and mice by injecting 1 : 2 : 5 : 6-dibenzanthracene dissolved in lard into the subcutaneous tissues. These tumours conformed to the usually accepted criteria of malignancy; grafted strains have now reached the 23rd and 36th generation in mice and the 18th and 20th generation in rats.

Technique.

The present paper sets out some results of the intraperitoneal injection of fatty solutions of 1 : 2 : 5 : 6-dibenzanthracene. This substance was dissolved in a concentration of 0.4 per cent. in olive oil by heating at temperatures not exceeding 100° and the solution was then emulsified in 3 volumes of 5 per cent. gum acacia in water usually made alkaline to p_H 8.2 in order to assist emulsification. The concentration of the hydrocarbon in the emulsion was thus 0.1 per cent.*

Intraperitoneal injections of this emulsion were made once a week into the right hypogastrium of rats and mice. At the commencement of the experi-

* The preparation of this emulsion was kindly undertaken by the Crookes Laboratories (British Colloids, Ltd.), to whom we are greatly indebted.

ments the rats received 0·5 c.c. and the mice 0·05 c.c., these doses being soon raised to 1 c.c. and 0·1 c.c. respectively.

The original purpose of this experiment was to cause the formation of a tumour in a part of the animal's body remote from the site of the injection of dibenzanthracene. It was thought that if this substance entered the general blood stream it might become subsequently localised and concentrated in an area of focal inflammation artificially produced, and so bring about a new growth in the inflamed tissue. Accordingly various substances (*e.g.*, starch, peptone broth) were injected into the napes of these rats and mice to set up an aseptic inflammation in this region. In no instance has a tumour occurred in the inflamed nape, but several of the animals have already succumbed to peritoneal tumours.

The account which follows is concerned mainly with a group of 10 rats which had been given weekly intraperitoneal injections of the emulsion of 1 : 2 : 5 : 6-dibenzanthracene described above, 2 c.c. of peptone broth being introduced into the nape of the neck on the same occasions, fig. 1.

Results.

The administrations were commenced on May 22, 1931, and the last rat was killed in the 49th week of the experiment. Eight of these 10 rats developed definite peritoneal tumours. Of the remaining two, one, rat No. 2, was killed in the 26th week with a large liver and ascites, and showed a condition of peritoneal thickening which suggested that tumour formation might have occurred later had the animal lived long enough. The remaining rat, No. 5, died and was eaten by its fellows, so that no post-mortem examination was possible.

The average life of the eight tumour-bearing rats after the commencement of the experiment was 40 weeks, the shortest life being 23 weeks, during which period 21 injections were given, and the longest life 49 weeks. The total amount of 1 : 2 : 5 : 6-dibenzanthracene administered in the former case was approximately 20 mg. and in the latter 43 mg.

The accompanying chart shows the history of these animals in graphic form (fig. 1).

The records of this group of rats are briefly as follows :—

Rat 1.—Killed in the 23rd week of the experiment. The rat at this time appeared ill and an abdominal tumour could be recognised during life. P.M. examination showed a rounded mass in which the cæcum, intestines and mesentery were inextricably bound. The mass, which was freely movable within the limits allowed by the mesentery, had no adventitious attachments to the abdominal

wall or to the liver, stomach or other viscera. No other tumours were observed. Microscopical examination showed the tumour to consist of polymorphic cellular tissue in which fusiform and oval cells predominated. Some giant cells were present. In one place the growth had invaded the intestine, and destroyed the muscular coats.

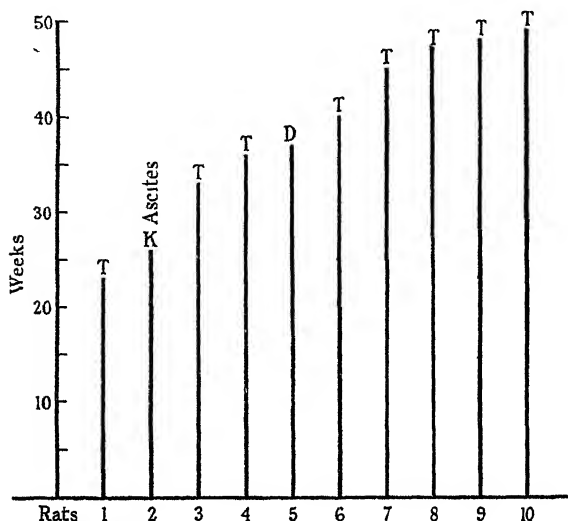


FIG. 1.—The numbers 1–10 below the base line are the reference numbers of the rats. Each vertical line represents the duration in weeks of the life of one rat from the commencement of the experiment. T = peritoneal tumour present at death. For rats 2 (K), and 5 (D), see text, pp. 240, 241.

Rat 2.—This animal was killed in the 26th week of the experiment on account of sanguineous ascites. P.M.—The liver was found to be large and pale, but no tumour was found. In places the peritoneum showed opaque patches, which under the microscope were seen to consist of a cellular layer derived apparently from proliferation of the endothelium. Subsequent observations suggest that this peritoneal thickening may have been a pre-cancerous condition.

Rat 3.—Killed in the 33rd week of the experiment on account of ascites and palpable abdominal tumours. P.M. showed: (1) a smooth, rounded abdominal tumour growing from the peritoneum in the left flank and unattached to the viscera; (2) another tumour lying between and incorporating the right kidney and liver; (3) several small nodules of growth in the peritoneum covering the intestines and accessory genital organs; (4) several thickened, opaque patches in the parietal peritoneum of the right flank; (5) a peritoneal tumour in the right loin constricting the ureter and causing hydronephrosis. Microscopical examination showed that the spindle-celled mass incorporating the liver and kidney had not invaded these organs, though it had invaded the pancreas. The peritoneal tumour in the left flank had infiltrated the subjacent abdominal muscles.

Rat 4.—Killed in the 36th week on account of gastric distension. Under anaesthesia stomach contents gushed from the animal's mouth without any of the systemic

muscular movements commonly associated with vomiting. P.M.—The stomach and duodenum were greatly distended owing to involvement of the jejunum in a hard mass of growth which had incorporated the gut with the liver. There were present also tumours of the parietal peritoneum around the sites of the needle punctures, and again on the left side near the attachment of the diaphragm; opaque, thickened plaques were scattered over the peritoneum. Microscopical examination showed that some of the peritoneal tumours had begun to invade the subjacent muscles, figs. 2 and 3, Plate 11. The opaque plaques represent thickening of the peritoneum which, instead of consisting of a single layer of cells, may be a millimetre or more in depth. The liver, fig. 4, Plate 11, and the muscular coat of the jejunum had been invaded by growth.

Rat 5.—This rat died in the 37th week and was almost entirely eaten by its fellows, so that no post-mortem observations could be made.

Rat 6.—Killed in the 40th week on account of abdominal distension. P.M.—A tumour had bound the pyloric portion of the stomach, the liver and pancreas into one mass, so as to cause gastric obstruction. Microscopically the growth consisted chiefly of spindle cells. It did not appear to have penetrated the capsule of the liver or the muscular coats of the stomach, though the pancreas had been invaded. No other tumours were present. Patches of thickened peritoneum were noticeable as in the former cases.

Rat 7.—Killed in the 45th week on account of ascites and debility. P.M.—(1) A large mass of tumour surrounded the pyloric portion of the stomach, fig. 5; (2) another mass surrounded and concealed the cæcum; (3) the manubrium sterni was embedded in tumour; (4) both halves of the diaphragm were infiltrated with growth; (5) the spleen was partially embedded in and fixed to the mass which involved the stomach. In addition to these growths there were numerous nodules and plaques in the peritoneum. Microscopical sections showed invasion by growth of the stomach, fig. 6, Plate 12, the intestine and the pancreas, fig. 7.

Rat 8.—Killed in the 47th week with sanguineous ascites. Tumours were present in the stomach—both pyloric and cardiac portions—the diaphragm, liver and the adnexa of the genital organs. A peritoneal tumour had compressed the ureter and caused hydronephrosis of the right kidney. Numerous plaques and nodules of growth were scattered about the peritoneum. Microscopical examination showed invasion by tumour cells of the diaphragm, stomach, liver, and the muscles of the abdominal walls, fig. 8.

Rat 9.—Killed in the 48th week with ascites. There was a mass spreading between the viscera of the anterior part of the abdomen binding together the lobes of the liver, the stomach, pancreas, spleen and left kidney. Microscope slides showed early invasion of the pancreas. The other viscera had not been penetrated by growth. Numerous plaques were present on the parietal peritoneum, the mesentery of the bowel and the under surface of the diaphragm. The muscle of the diaphragm was infiltrated by growth, and some of the other peritoneal nodules had spread outwards amongst the superjacent muscle fibres.

Rat 10.—Killed in the 29th week on account of ascites and debility, fig. 10. P.M.—Tumours were present involving the diaphragm, liver, pancreas, stomach,

mesentery, intestine and peritoneum. The right kidney was hydronephrotic from compression of the ureter by growth. Microscopical examination showed that the diaphragm had been perforated, fig. 9, and there was invasion of the pancreas, liver, stomach, intestine and abdominal wall.

The distribution of the abdominal tumours produced in these rats is shown in a general manner in Table I.

The relative frequency with which tumours have occurred in the diaphragm is noteworthy and depends upon the distribution of the emulsion after its introduction into the abdomen. Observation has shown that an emulsion of fat, like a colloidal dye, when injected into the abdomen of a rat or a mouse, tends to pass gradually forward to the anterior portion of the cavity. In order to obtain a demonstration of this distribution a rat was given 1 c.c. of lipiodol into the right posterior quadrant of the abdomen. An X-ray photograph taken 37 days later showed that a considerable proportion of the injected material had become applied to the under surface of the diaphragm.

These peritoneal tumours are of much interest, for the development of a malignant neoplasm can be observed in every stage. The first visible response is a patchy opacity occurring here and there in the peritoneum. This opacity is due to a thickening of the membrane either by a proliferation of its own cells or by a deposit of cells upon its surface. As this thickening progresses plaques can be detected, and also nodules which give the peritoneum a warty appearance. Microscopical examination shows that many of these nodules are accompanied by a malignant infiltration of the subjacent voluntary muscle, figs. 2, 3, 8; and it has been noticed on several occasions that even in the absence of a nodule, a mere plaque is already associated with invasion of the underlying tissues. The general type of the tumours is spindle-celled, but giant cells are frequently seen, fig. 9, and occasionally there is a pronounced polymorphism. Tumours with these different cell characteristics may coexist in the same animal.

In no case has any metastasis in extraperitoneal regions been observed. Direct extension of a neoplasm into the pleural cavity through the diaphragm has occurred in two instances. The fibrous capsules and septa of the organs appear to offer the most resistance to the neoplastic invasion, but once this barrier has been passed the other tissues appear to oppose but little resistance.

Other groups of rats and mice have been treated in the same way as those of which details have been given above, but in the majority of these the experiment was started at a later date than in the completed series which forms the subject of this paper. Some abdominal tumours have occurred already in

Table I.—The Distribution of Tumours in the Abdomen.

	Mass around caecum, intestine and mesentery.	Parietal peritoneum.	Pyloric region of stomach and pancreas.	Diaphragm.	Around r. ureter causing hydro- nephrosis.	Root of mesentery.	Genital adnexa and pelvic organs.	Other situations.	Ascites present.
Rat 1	+	+			+		+	r. kidney and liver	+
Rat 3	+	+				++			
Rat 4	+	+							
Rat 6	+	+	+	+	+		+	liver	++++
Rat 7		+	+	+	+			liver	
Rat 8		+	+	+	+				
Rat 9		+	+	+	+				
Rat 10	+	+	+	+	+				

these later groups and are available for further study. The results up to the present date of these additional experiments are given in Table II.

Table II.

Animals.	Number.	Dead without tumours.	Dead with tumours.	Still living.
Rats	80	27	10	43
Mice.	120	80	17	23

Control Experiments.

A control series of 20 rats is under observation which are receiving intraperitoneal injections weekly of the same olive oil, in the unemulsified state. At the time of writing (184th day of experiment) no tumours have been detected in the animals.

A difficulty arises in such investigations, in that the control animals bearing no tumours live very much longer than others which develop tumours, and the experiment cannot be reported as complete until the last control animal is dead, which may be a matter of years. Hence in the earlier paper upon subcutaneous spindle-celled tumours, 46 in all, in mice and rats following injection *sub cutem* of 1 : 2 : 5 : 6-dibenzanthracene in lard (Burrows, Hieger and Kennaway, 1932) we laid stress upon the fact that we were not yet prepared to define exactly the part played by the fat in the experiments described. The present state of these control experiments may be stated here.

(1) One hundred and thirty mice have been injected with the lard, and with the various mixtures of milk and lard which were being used at the same time as solvents of 1 : 2 : 5 : 6-dibenzanthracene. Early in the course of the experiments three of these animals in one series of 10 developed spindle-celled tumours at the site of injection; grafts from one of these failed to take. Suspicion at once arose that some confusion of the animals, or of the substances injected, in the control and other series had occurred. Since more complete precautions against any such mishap have been taken no further tumours have arisen in the controls, and no tumours have appeared except in this one series. Unfortunately, there was a high death rate among these mice, but 40 lived for more than 120 days, and 7 are alive at the time of writing (482nd day).

(2) Twenty-five rats have received frequent injections in the groin of lard

which had been sterilised at 100°; 20 of the animals are now alive (382nd day) and no tumours have been obtained.

(3) Injections in the groin have been made in mice (numbers given in brackets) of the following fats and oils; fresh pork fat (50), olive oil (10), sperm oil (10), cod liver oil (10). Some of these animals have lived for more than 400 days and no tumours have been obtained.

(4) In a further series of experiments* six spindle-celled tumours were obtained from a series of 10 rats injected *sub cutem* with 1 : 2 : 5 : 6-dibenzanthracene in lard, the last animal being killed on the 216th day; while a control series of 10 rats receiving the same lard alone had produced no tumours at the date of writing (297th day), when nine animals are still alive.

Lard heated to higher temperatures may have some neoplastic action, and further experiments upon this matter will be reported.

Discussion.

Tumours of connective tissue have now been produced at this Institute in the eight ways shown in Table III.

Table III.

Animal.	Hydrocarbon.	Solvent.	Site of injection.
1. Rat	1 : 2 : 5 : 6-dibenzanthracene	Lard	<i>Sub cutem.</i>
2. Mouse	”	”	”
3. Rat	”	Olive oil emulsion	”
4. Rat	”	”	Peritoneum.
5. Mouse	”	”	”
6. Mouse	”	Lard	”
7. Fowl	”	”	<i>Sub cutem.</i>
*8. Mouse	5 : 6-cyclo-penteno-1 : 2-benzanthracene	”	”

In connection with these results it is necessary to consider what part, if any, may have been played in the carcinogenesis by the fatty media as distinct from the dibenzanthracene. For the present, and pending the outcome of the control experiments mentioned above, the question whether the introduction of lard alone or of an emulsion of olive oil alone into the subcutaneous tissues or the peritoneum of a rat or mouse can determine the development of

* Unpublished work by J. W. Cook and G. Barry.

a malignant neoplasm, must remain open. It is, of course, quite possible that a tumour may be produced by fat alone in a susceptible animal. Cori (1927) has recorded a spindle-celled tumour arising in the neighbourhood of a sub-cutaneous injection of "oil" containing ovarian hormone in a mouse of a strain liable to the development of such neoplasms. If it were found possible to produce a large yield of tumours by the action of any fat which might occur in the body, this result would be of greater interest than any which could be obtained with synthetic hydrocarbons. At present we can do no more than record the abundance of tumours which have been obtained by the use of 1 : 2 : 5 : 6-dibenzanthracene in fatty media.

Summary.

(1) Experiments are recorded in which numerous spindle-celled tumours were induced in rats and mice by the intraperitoneal injection of 1 : 2 : 5 : 6-dibenzanthracene dissolved in olive oil. The characters of the tumours are described.

(2) The respective parts played by the hydrocarbon and fat in the carcinogenesis have been left undetermined.

I wish to express my indebtedness to W. Davis, who has prepared the very numerous histological specimens, and to F. Goulden and E. L. Butler for the photographs which accompany this paper.

EXPLANATION OF PLATES.

PLATE 11.

FIG. 2.—Rat 4. Whole thickness of abdominal wall, from skin (below) to peritoneum (above), showing spindle-celled nodule invading voluntary muscle. $\times 4.5$.

FIG. 3.—Rat 4. Edge of peritoneal tumour shown in fig. 2. $\times 24$.

FIG. 4.—Rat 4. Invasion of liver by tumour. $\times 84$.

FIG. 5.—Rat 7. Tumour (T) surrounding oesophagus (O) and invading wall of stomach (S). L = liver. $\times 3$. See fig. 6.

PLATE 12.

FIG. 6.—Rat 7. Invasion of walls of stomach by tumour. $\times 21$.

FIG. 7.—Rat 7. Early invasion of pancreas by tumour. $\times 21$.

FIG. 8.—Rat 8. Invasion of voluntary muscle of abdominal wall. $\times 84$.

FIG. 9.—Rat 10. Giant-celled tumour invading diaphragm. $\times 84$.

PLATE 13.

FIG. 10.—Rat 10. Tumours of parietal peritoneum (P), diaphragm (D) [shown in fig. 9], mesentery (M). E = residue of emulsion injected.

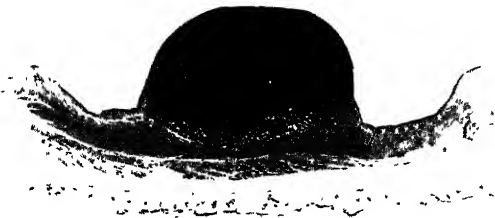


FIG. 2.



FIG. 3.



FIG. 4.

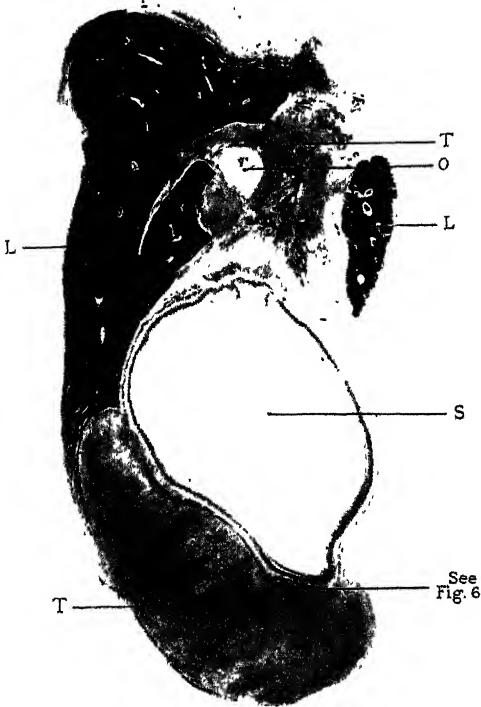


FIG. 5.



FIG. 6.



FIG. 7.



FIG. 8.

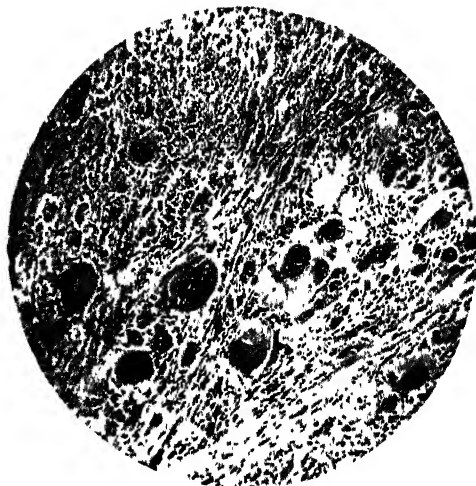


FIG. 9.



FIG. 10.

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*Further observations on the Medulla oblongata of Cyprinoids; and
a comparative study of the Medulla of Clupeoids and Cyprinoids
with special reference to the Acoustic tubercles.*

By H. MUIR EVANS.

(Communicated by Sir Henry Dale, Sec. R.S.—Received May 10, 1932.)

Introductory.

In a previous paper (Evans, 1931) an endeavour was made to correlate the feeding habits of Cyprinoids with the conformation of the medulla oblongata, and it was concluded that the Cyprinoids can be divided into three main groups.

It was found:—

- (1) That the vagal lobes are large in the Carps, the Goldfish and the Bream and that these are all mud feeders.
- (2) That the vagal lobes and the facial lobe are small in the Roach, the Rudd, the Chub and the Dace, all of which take a fly and feed largely by sight.
- (3) That the facial lobe is large in the Gudgeon, the Barbel and the Tench. The facial nerve in this group, all of which possess barbels, divides into two branches after entering the medulla oblongata. The Gudgeon and the Barbel grope and grub for their food and never take a fly.

The Tench, however, has feeding habits not unlike those of a Carp; in fact, it may be regarded as being a transitional form between the types (1) and (3).

Through the kindness of Mr. Michael Graham, naturalist on the staff of the Ministry of Agriculture and Fisheries, I have had the opportunity of

examining a specimen of *Engraulicypris argenteus* from the Victoria Nyanza. It is an interesting fact noted by Graham that this fish has ceased to behave like a Cyprinoid and has become solely a plankton-feeder. Its name has an obvious reference to its habits and appearance, Engrauli: cypris, or the Anchovy-carp.

The following are extracts from his Report (Graham, 1929) on the Victoria Nyanza and its Fisheries :—

“ So far as the evidence goes this fish resembles the Clupeoid fishes in its pelagic or open water habitat as well as in its structure.” “ We frequently observed *Engraulicypris* apparently catching Copepods and other members of the plankton near the surface. The stomachs contained Cladocera or Copepoda.”

“ This is an interesting example of a fish belonging to a typically river family, the Cyprinoids, taking on a very different form where the conditions resemble those of the sea, especially in the abundance and stability of a rich population of plankton, and adopting not only a pelagic existence, but the shape and appearance of a pelagic family.” The resemblance to marine fish does not end here : Mr. Graham also found “ some floating segmenting eggs in the plankton,” which he gives evidence to prove were the eggs of *Engraulicypris*, and says “ So far as I know this is the first record of a floating egg in fresh-water fish. Many sea fish have floating eggs.”

This statement may be true as regards large fresh-water lakes, but *Ophiocephalus* has floating eggs ; it clears a hole in the reeds at the edge of a swamp and the eggs float in the protected pool so formed.

It appeared to be a possibly fruitful research to examine the medulla oblongata of this fish in order to see whether, associated with its habit of plankton feeding, there was any difference in type of medulla oblongata from those Cyprinoids we have already examined. That this is the case will appear from an examination of serial sections of the medulla oblongata, stained by the same method as we have employed throughout, namely, staining with hæmatoxylin and counter staining with picro-indigo-carmin.

The Medulla Oblongata of Engraulicypris.

A naked eye examination at once reveals the fact that there is no facial lobe appearing in the usual position, not even a small facial as is found in the

Roach group. Serial sections, figs. 1-6, show that the vagal lobes are small, fig. 1. As one travels forwards the dorsal portions of the vagal lobes increase

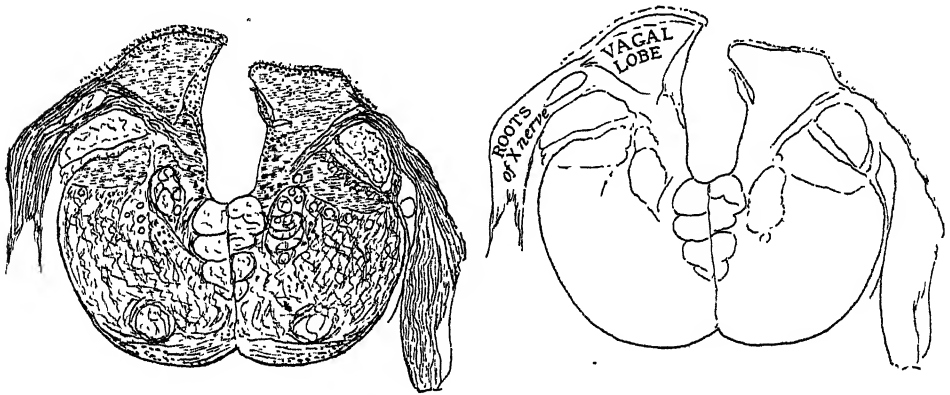


FIG. 1.—*Engraulicypris*.

in size so that they appear club-shaped, with the thicker portion tending to bulge towards the middle line, fig. 2. The next change to be seen is the union of the two club-shaped dorsal portions in the middle line. From this united portion descending fibres are seen to pass downwards and outwards on either

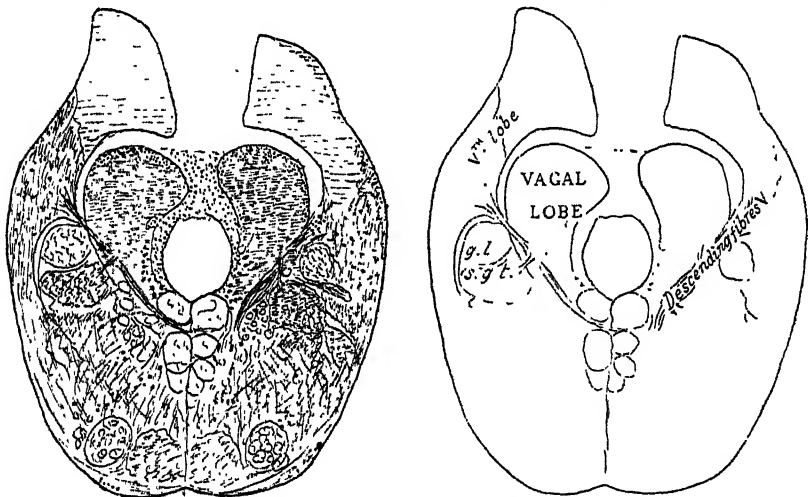


FIG. 2.—*Engraulicypris*.

side into the great longitudinal secondary gustatory tracts, so that we may assume we have now reached the posterior portion of the facial lobe, which is completely hidden and overlapped by the Vth lobes which are highly developed and meet in the middle line, fig. 3.

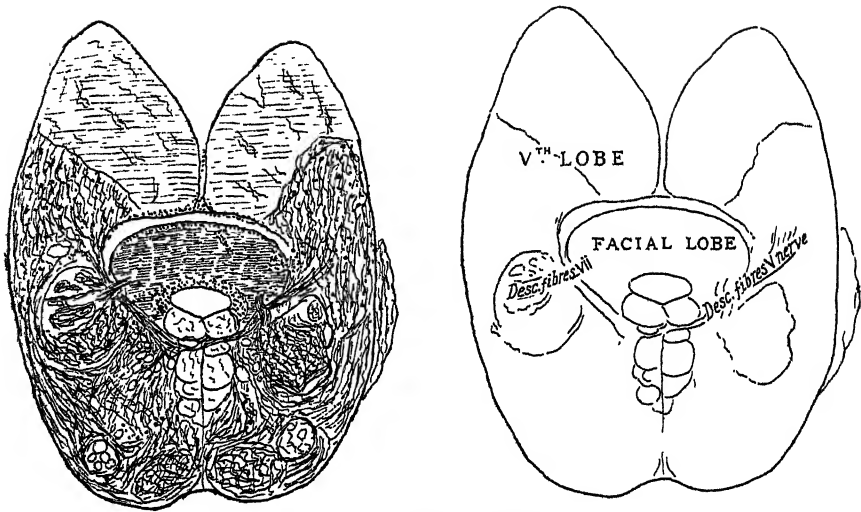
FIG. 3.—*Engraulicypris*.

Fig. 4 shows the commencement anteriorly of the two facial lobes before they coalesce and the facial nerves are seen within their substance in section. Nerve fibres are also to be seen passing to the great longitudinal tracts.

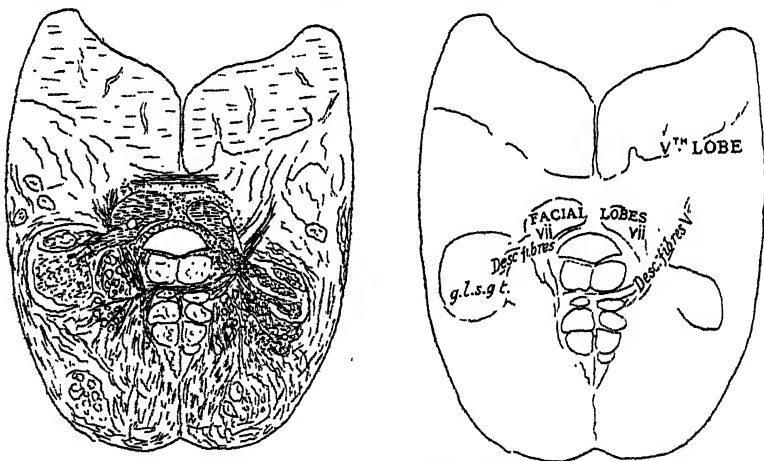
FIG. 4.—*Engraulicypris*.

Fig. 5 shows the facial nerves in section passing backwards in a longitudinal direction and lying just exterior to the ventricle. The commencement of the cerebellum is shown dorsally and lying at the inferior surface of its base are seen groups of small-celled tissue arranged in radiating bands passing upwards and outwards. These bands are separated by strands of nerve fibres, which meet and form a transverse band inferiorly.

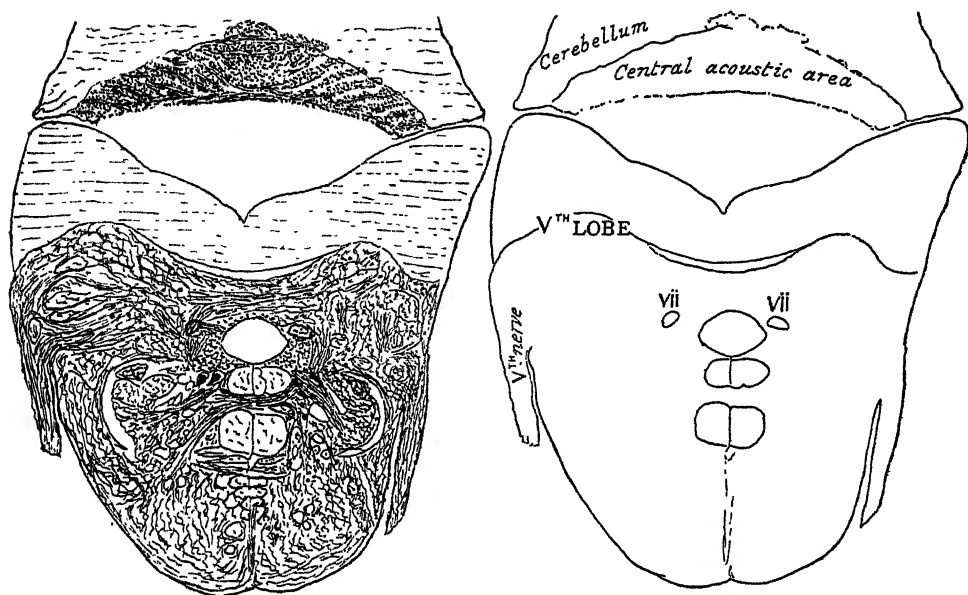


FIG. 5.—*Engraulicypris*.

Fig. 6 shows the facial nerves entering the medulla oblongata transversely. The characteristic section of a Cyprinoid cerebellum is shown and on the right

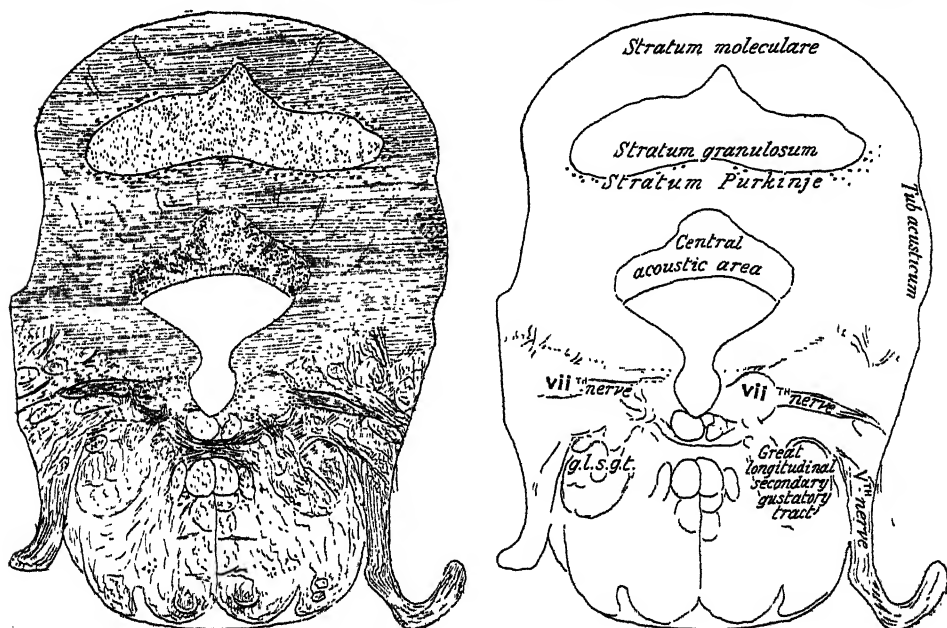


FIG. 6.—*Engraulicypris*.

side (apparent) is seen the commencing protuberance of the acoustic tubercle, and the beginning of the small-celled tissue characteristic of this lobe. It may be well to note that in using the term "acoustic tubercles," the traditional practice is being followed. But in view of the fact that they are mainly vestibular in function and that the auditory function is at least a matter that is still under discussion, we would suggest that the term "octaval tubercles" would be more appropriate, as such a term would not be subject to the same criticism and possible ambiguity as the word "acoustic" involves. However, throughout this paper we propose using the term "acoustic tubercles," having made it clear that in our opinion this terminology should be superseded. At the inferior margin of the cerebellum there appears a triangular mass of tissue; a continuation of that seen in the same area in the proceeding section. This is seen to be connected by nerve fibres, passing outwards, with the acoustic tubercle in sections at a more anterior level.

The significance of this tissue will be discussed at the close of this paper. For the present we can sum up the peculiar characteristics of the medulla of *Engraulicypris* as (i) the presence of small vagal lobes which unite medially and are continuous anteriorly with a flattened facial lobe. The latter results from a fusion of two small facial lobes, and (ii) the complete enclosure of the facial lobe by the highly developed Vth lobes.

It appeared necessary to examine the brain of a marine plankton feeder to see whether the picture of its medulla oblongata would in any way approach the type just described, which obviously does not conform to any one of the groups I, II or III into which we have been able to place the medulla oblongata of other Cyprinoids. Before doing so, however, we investigated the medulla of the Bleak, *Alburnus lucidus*, because in the Swiss lakes such as Lake Geneva, and the French lake Annecy, the Bleak seems to be largely a surface feeder. According to Burne (1902) the brain of the Bleak closely resembles that of the Tench except in the somewhat smaller relative size of the *lobus impar* (facial lobe).

Our observations disagree with this description of the naked eye appearance. We note a very small facial lobe, large acoustic tubercles, and a curious displacement of the vagals. Moreover, the microscopic structure appears to differ very markedly from that of the Tench; in fact, a series of sections of the medulla oblongata show a very marked similarity to the condition found in *Engraulicypris*.

Figs. 7 to 13.—Sections of the medulla oblongata of the Bleak can be now compared with figs. 1 to 6 of *Engraulicypris*. The vagal lobes in the Bleak become

club-shaped, in fig. 7, and in fig. 8 are seen to join. In fig. 9 a small spherical facial lobe appears at the dorsal junction of the vagal lobes, and fibres are seen

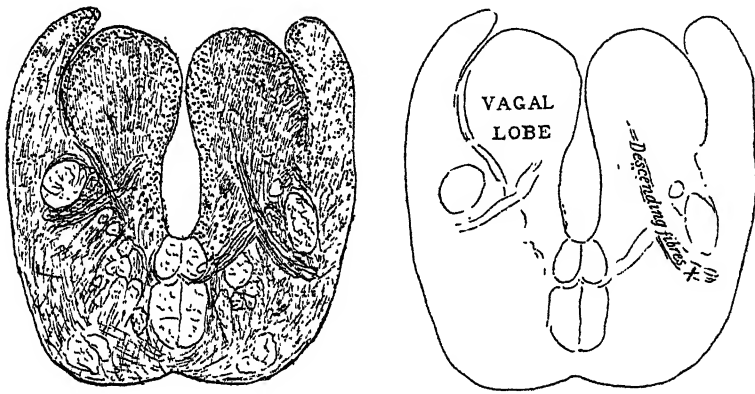


FIG. 7.—Bleak.

to pass downwards from it through the vagal lobes to the great longitudinal secondary gustatory tracts. The facial lobe just appears between the Vth lobes. In fig. 10 the commencement anteriorly of the facial lobe is seen, and

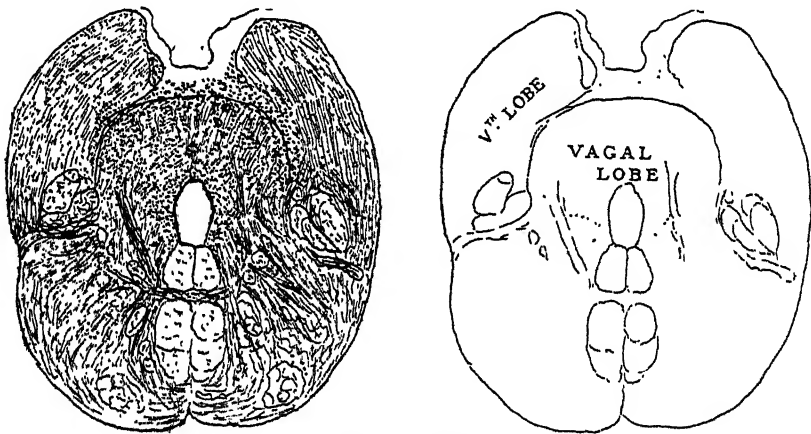


FIG. 8.—Bleak.

on either side the facial nerves appear in cross section. On the right descending fibres pass to the great longitudinal tracts.

In fig. 11 the facial nerves are seen in cross section as they pass backwards. In fig. 12 they appear entering the medulla transversely. So that it would seem that the Bleak's medulla oblongata is a transitional form between that of a Roach and *Engraulicypris*.

We would now draw attention to the central mass of cells lying at the base of the cerebellum near its origin. We notice the radiating groups of cells and nerve fibres in fig. 11 forming a somewhat triangular mass extending beyond

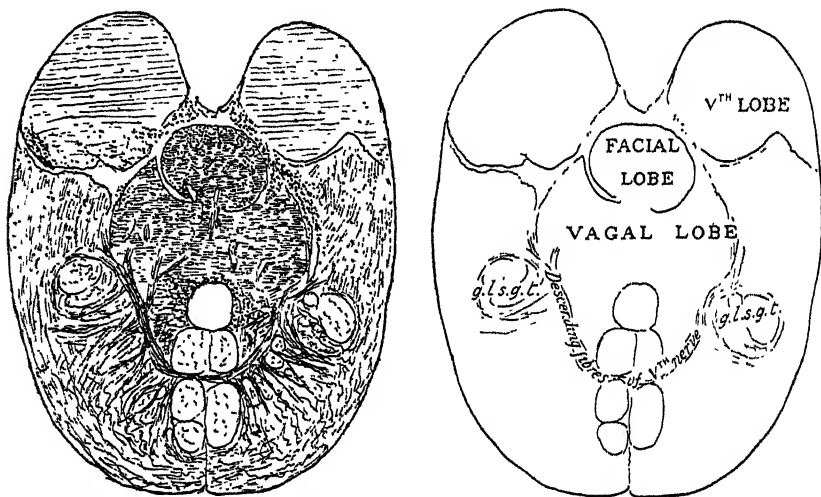


FIG. 9.—Bleak.

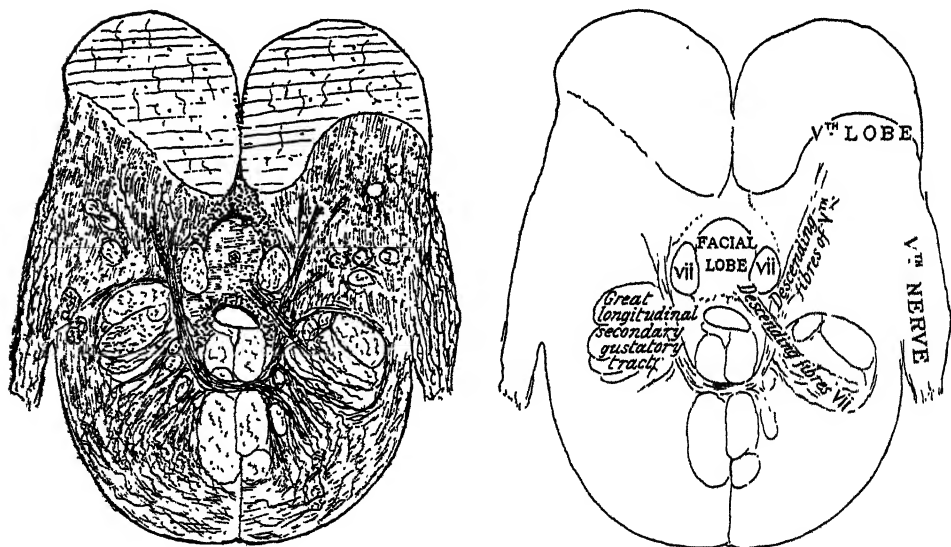


FIG. 10.—Bleak.

the limits of the medulla oblongata laterally and in fig. 12 the triangular shape of this area alters, as a deep indentation divides it into a right and left portion. From the margins of this cleft the cells pass outwards separated by nerve

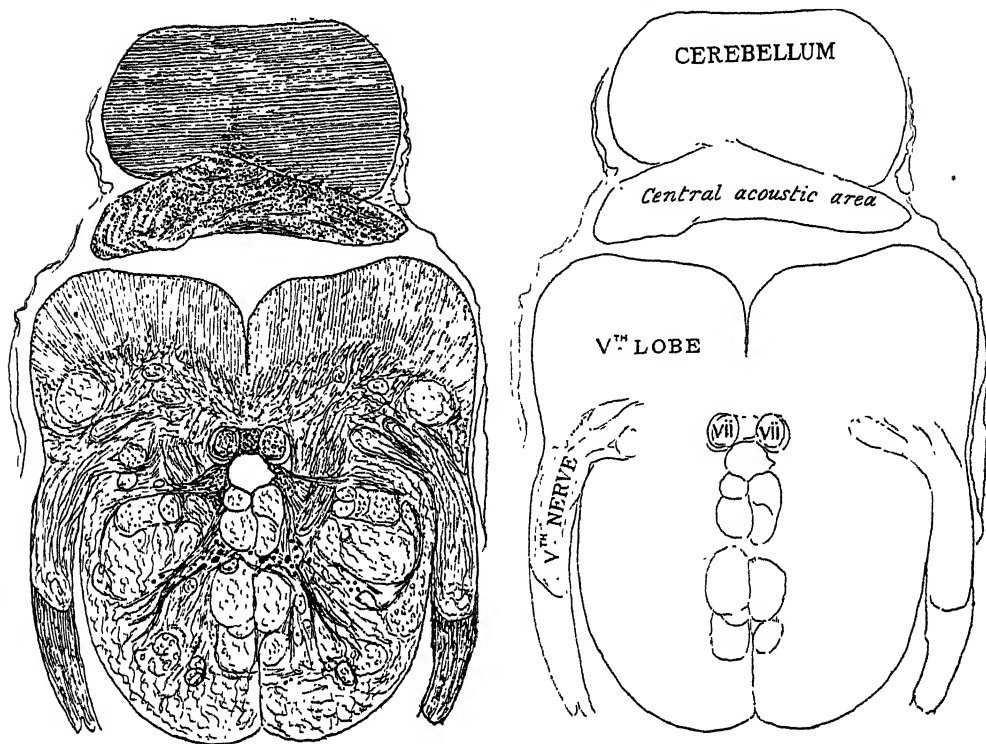


FIG. 11.—Bleak.

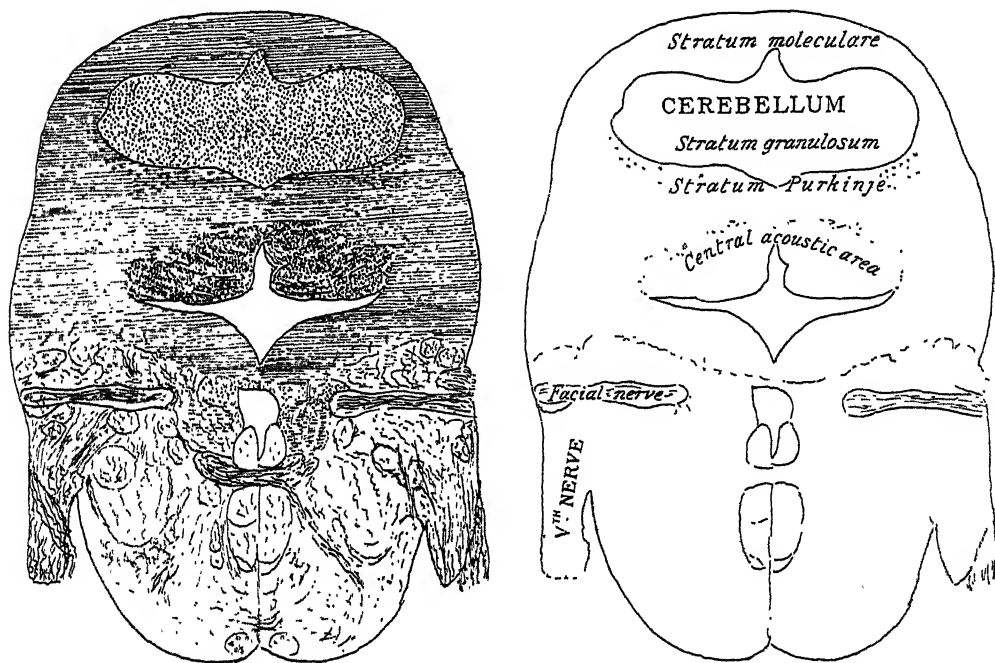


FIG. 12.—Bleak.

fibres running in a transverse direction. We would draw special attention to fig. 12A a section at the level of the acoustic tubercles. This detailed drawing shows fibres connecting the central area with the accumulation of cells forming the acoustic tubercles from which descending fibres are seen to pass downwards and inwards. The central mass of cells has gradually diminished in size, but the lateral groups of cells are still connected by transverse fibres. The importance of this central area will appear when we study the same area of

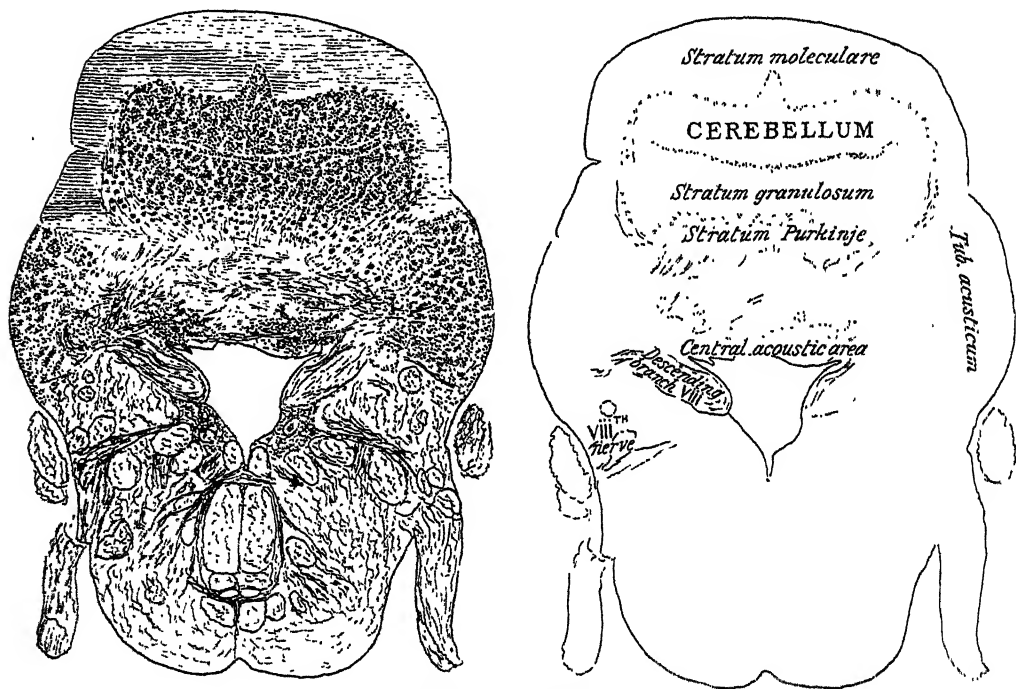


FIG. 12A.—Bleak.

the brain in Clupeidae and compare its relative development in the various species of the Cyprinidae.

It appears from the above study of the medullæ oblongatæ of *Engraulicypris* and the Bleak that we are justified in describing a fourth group of Cyprinoids, classified according to their type of medulla oblongata. It is an interesting fact that, in addition to the characteristic medulla of this fourth group, the external appearances are similar in these fishes.

According to Graham (1929) "*Engraulicypris* has small shining scales which rub off easily. These and its shape make it resemble whitebait." We find a high number of rays in the anal fin; in one specimen we counted 12 rays.

A heap of Bleak on the slab of a fishmonger's shop in Annecy recalled the appearance of a mass of small Herrings ; the fish lay in a mess of small scales and blood-stained mucus. The silvery scales of the Bleak are small and easily detached. According to Tate Regan (1911) this species *Alburnus lucidus* has 7-9 branched rays in the dorsal fin and 15-20 in the anal fin ; and the scales in the lateral line number 46 to 54. Specimens from Lake Annecy had 7 branched rays in the dorsal fin and 16-17 in the anal ; the scales in the lateral line numbered 52-54. The body is elongate, the coloration silvery white with the back a deep blue. Tate Regan (1911) in his classification of Ostariophysi, based on skeletal characters, places *Alburnus* next to *Abramis*, but its habits and medulla oblongata are very unlike those of the Bream.

The significance of the relative development of the various centres in the medulla oblongata can only be arrived at by a comparative study of several species of a family ; it is therefore profitable to compare two extreme types of the medulla oblongata in Cyprinoids and for this purpose we propose to select *Engraulicypris*, a plankton feeder, and the Loach, a purely ground feeder. The latter, though not strictly speaking a Cyprinoid, is a very near relative and, moreover, has an exaggerated development of the facial lobe of the type found in the Gudgeon.

The surface of this is lobulated and it widely overlaps the vagal lobes. The two diagrammatic sections, figs. 13, *a* and *b*, show the facial nerves, which

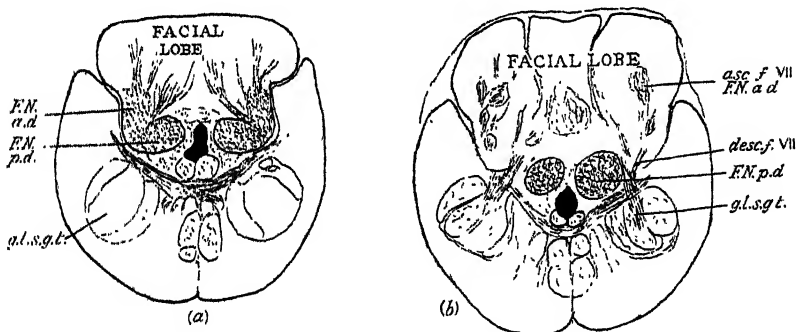


FIG. 13, *a* and *b*.—Loach. *FN.a.d.*, Facial nerve anterior division. *FN.p.d.*, Facial nerve posterior division. *asc.f. VII*, Ascending fibres VII. *desc.f. VII*, Descending fibres VII. *g.l.s.g.t.*, Great long. sec. gust. tracts.

are very large, dividing at the inferior surface of the lobe ; each anterior branch splits up into two groups of fibres, one passing centrally and the other laterally to the dorsal area of the lobe. The posterior branches are seen at their commencement passing backwards on either side of the ventricle in both figures ;

the lobules are shown and within them groups of anterior fibres. The posterior branches a little farther back (not shown) are found to enter the hinder end of the lobe ; on either side large descending fibres are seen entering the middle division of the great longitudinal secondary gustatory tracts. We now pass to a consideration of the acustico-lateralis area in the Loach. This will also be found to differ markedly from the condition found both in *Engraulicypris* and the Bleak. The central group of cells at the base of the cerebellum which is so marked in these fishes and which we have seen joins up with the acoustic tubercles does not appear ; it is true that there are a few cells forming a narrow band crossing from one side to the other, but there is no group of cells that could be described as a central acoustic area ; the band becomes rather more prominent at its external extremities, fig. 16, *q, r, s*, Loach. The acoustic tubercles are also feebly developed in the Loach when compared with those of *Engraulicypris* and the Bleak, fig. 15, *d, e*. So that in the latter group we note a rudimentary facial lobe and marked acoustic tubercles connected with a large central accumulation of cells at the base of the cerebellum, while in the Loach and Gudgeon we find a very large facial lobe and no central group of cells connected with the acoustic tubercles.

The Facial and Acoustic Lobes in the Herring as a Type of Clupeidæ.

The result of our investigation into the relative size of the vagal and facial lobes in *Engraulicypris* has shown that this fish has to a large extent lost the distinctive facial lobe of Cyprinidæ, and to a less extent the same picture is presented by the Bleak—a small facial lobe forming a small spherical mass at the anterior end of the united vagal lobes. To ascertain whether a similar configuration of the medulla oblongata appears in purely plankton feeders, it seemed desirable to examine the hind-brain of the Clupeidæ, and for this purpose the Herring and the Sprat appeared the most suitable fishes and the most easy to obtain. It was with some measure of surprise that the statement was read in the Museum Catalogue of the Royal College of Surgeons that “ the medulla of the Herring is much concentrated and has a well-marked facial lobe behind the cerebellum.” It is true that there is a well-marked lobe behind the cerebellum, but it is certainly not a facial lobe. We find on examining serial sections of the medulla oblongata of the Herring that resting on the Vth lobe appear two wings of tissue that meet dorsally, enclosing a small space, fig. 14, *f* and fig. 15, *k*. These wings increase in thickness, fig. 15, *g*, and fig. 17, and there soon appears a growth of tissue from the surface of the Vth lobe which

gradually surrounds this central mass, fig. 15, *g*, *h*, and fig. 18. These wings, fig. 17, when examined in object are found to consist of groups of round cells, through which nerve fibres pass dorsally to join at the apex, and a cortical layer of interrupted groups of round cells. This central lobe appears in section at first pyramidal in shape, but as the sections are followed anteriorly the lobe becomes more pear-shaped, fig. 18, the details of which can be followed in the drawing. A little farther forward the sections show the commencement of the cerebellum lying dorsal to the apex of the pear-shaped tissue, fig. 15, *h*, and at the lateral margins the acoustic tubercles commence to make their appearance, fig. 15, *i*. The acoustic and lateral line nerves are seen entering and a well-marked separate strand of fibres, which can be seen penetrating the medulla in fig. 15, *f-h*, appears at the base of the acoustic lobes.

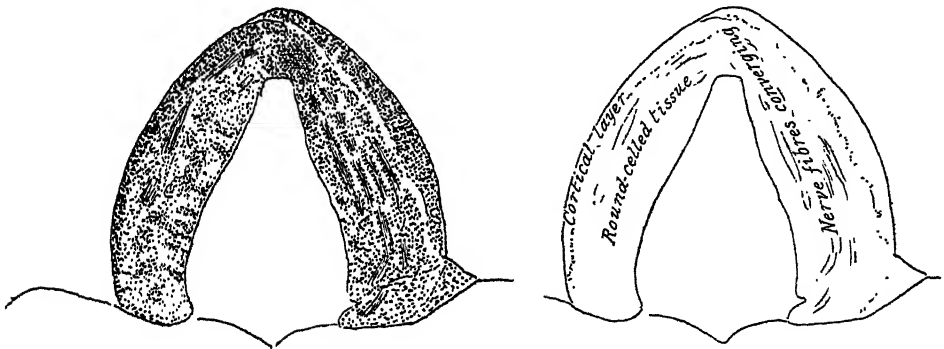


FIG. 14.—Herring. Central lobe.

Fig. 19 and fig. 15, *j*, show the central “acoustic” lobe sending fibres outwards to the acoustic tubercle and in fig. 15, *k*, to the right of the section, the acoustic tubercle is seen approaching the round celled central portion of the cerebellum.

Figs. 20 and 21 of the hind brain of the Sprat in the region of the acoustic tubercle show that a similar condition occurs in this fish. The sections have unfortunately been cut rather obliquely; nevertheless this error in technique is not altogether to be regretted as it shows, to the right, fig. 20, the central “acoustic” lobe lying free, and on the other side the acoustic tubercle, and fibres passing from the central lobe outwards. Also in fig. 20 the central lobe is found completely surrounded by tissue continuous below with the Vth lobe to the right of the section and on the other side the acoustic tubercle with the same definite strand of fibres as appears in the section of the Herring. In

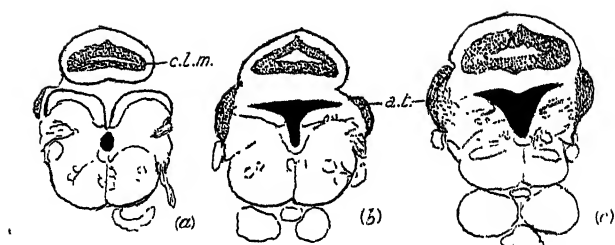
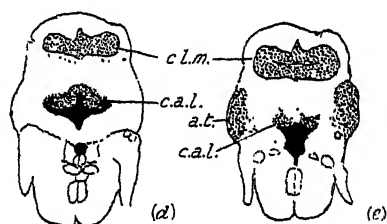
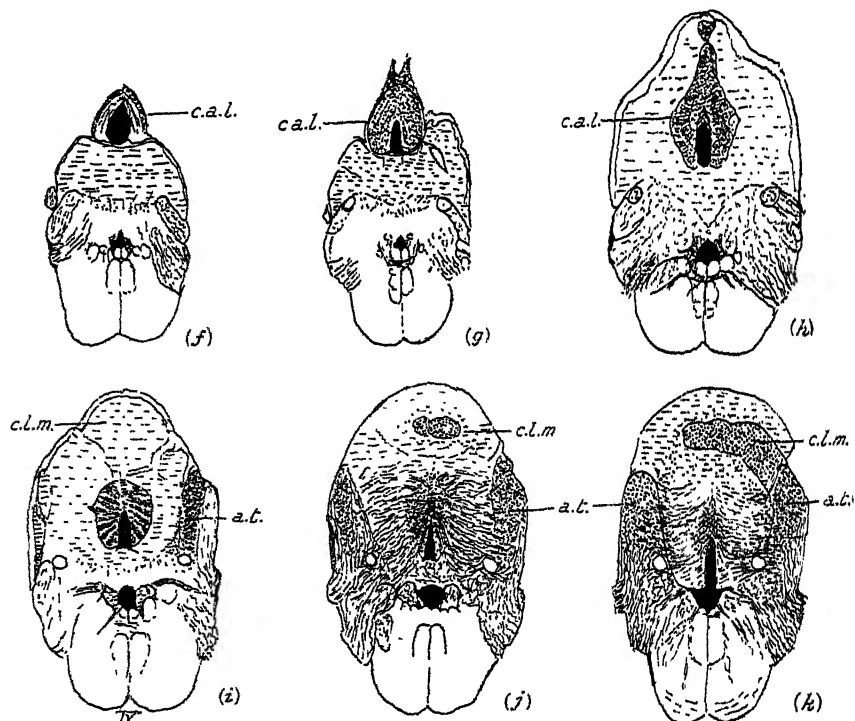


FIG. 15, *a-c*.—Whiting. *a*, Cerebellum overlapping medulla. To the left commencement of acoustic tubercle; *b* and *c*, cerebellum and acoustic tubercles.



d, e.—Bleak. Central acoustic area large. *d*, cerebellum and central acoustic area; *e*, cerebellum and acoustic tubercles.



f-k.—Herring. *c.a.l.*, Central acoustic lobe; *clm.*, cerebellum; *a.t.*, acoustic tubercles.

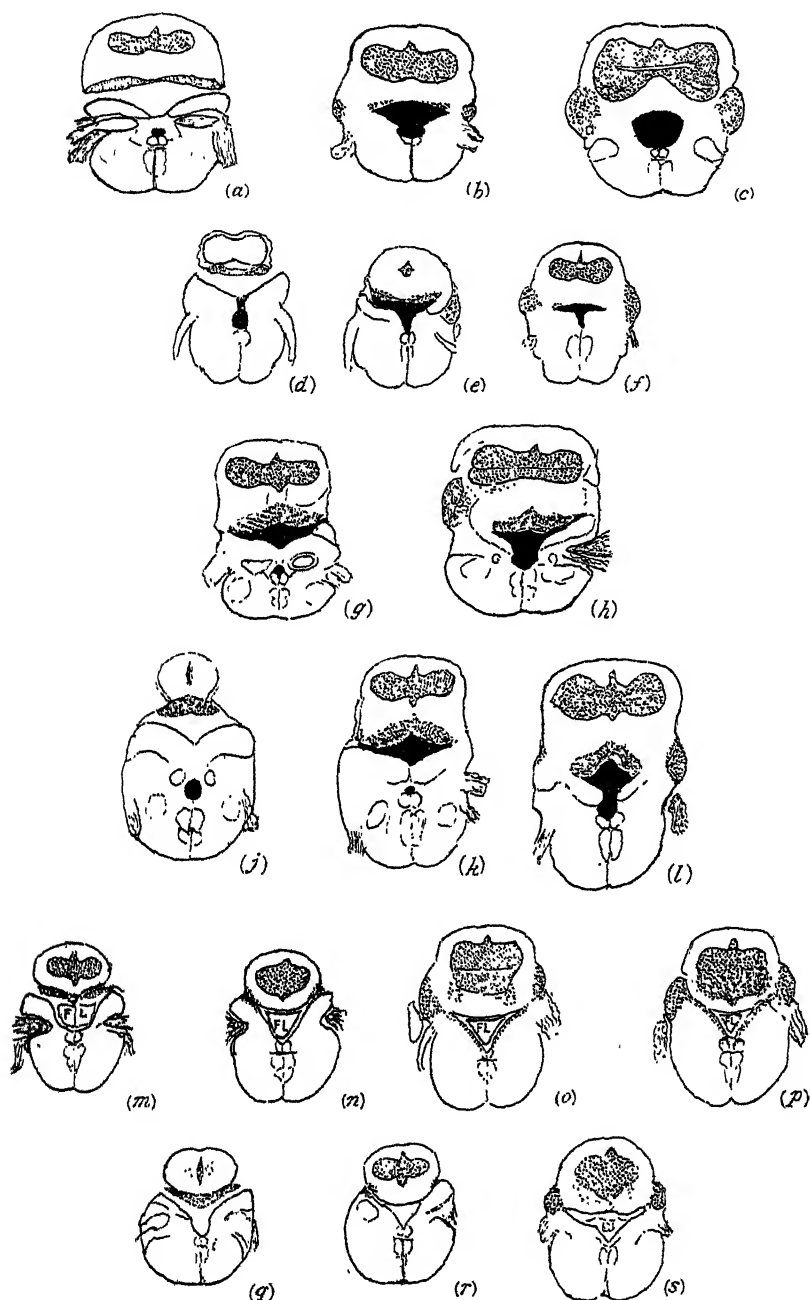


FIG. 16.—a-c, Tench. Small central acoustic area. d-f, Minnow. Small central acoustic area. g, h, Carp. Central acoustic area large. j-l, Roach. Central acoustic area large. m-p, Gudgeon. No central acoustic area. q-s, Loach. No central acoustic area.

this section the descending fibres of the Vth nerve passing downwards to the Vth motor nucleus and decussating are well shown. The next objective to be obtained was to trace the facial nerve of the Herring into the facial lobe. The facial nerve can be readily identified by its entrance at the level of the Vth nerve

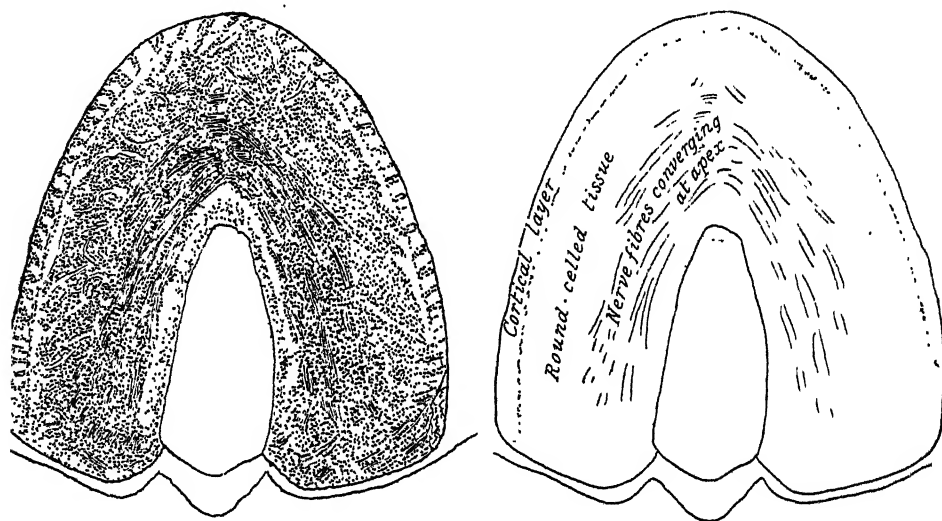


FIG. 17.—Central lobe of Herring.

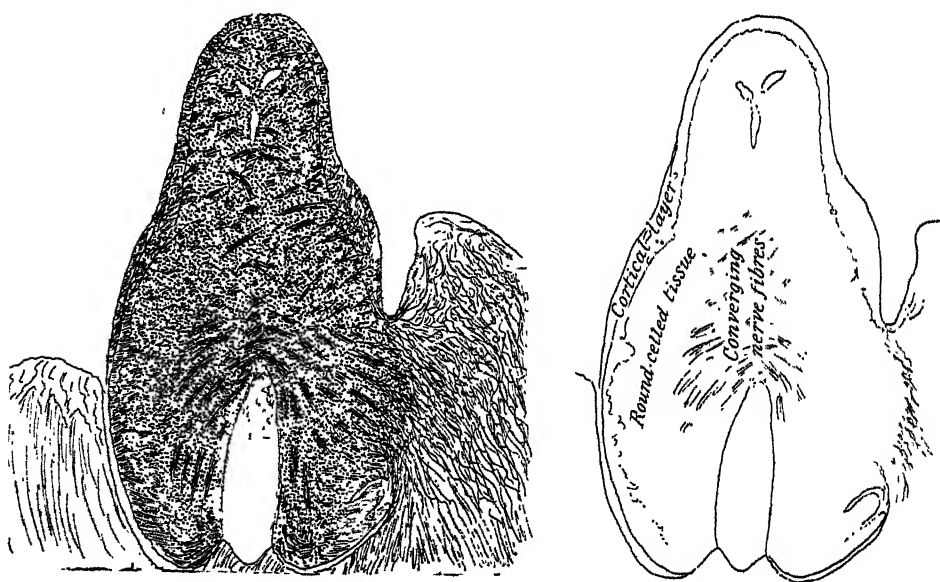


FIG. 18.—Central lobe of Herring.

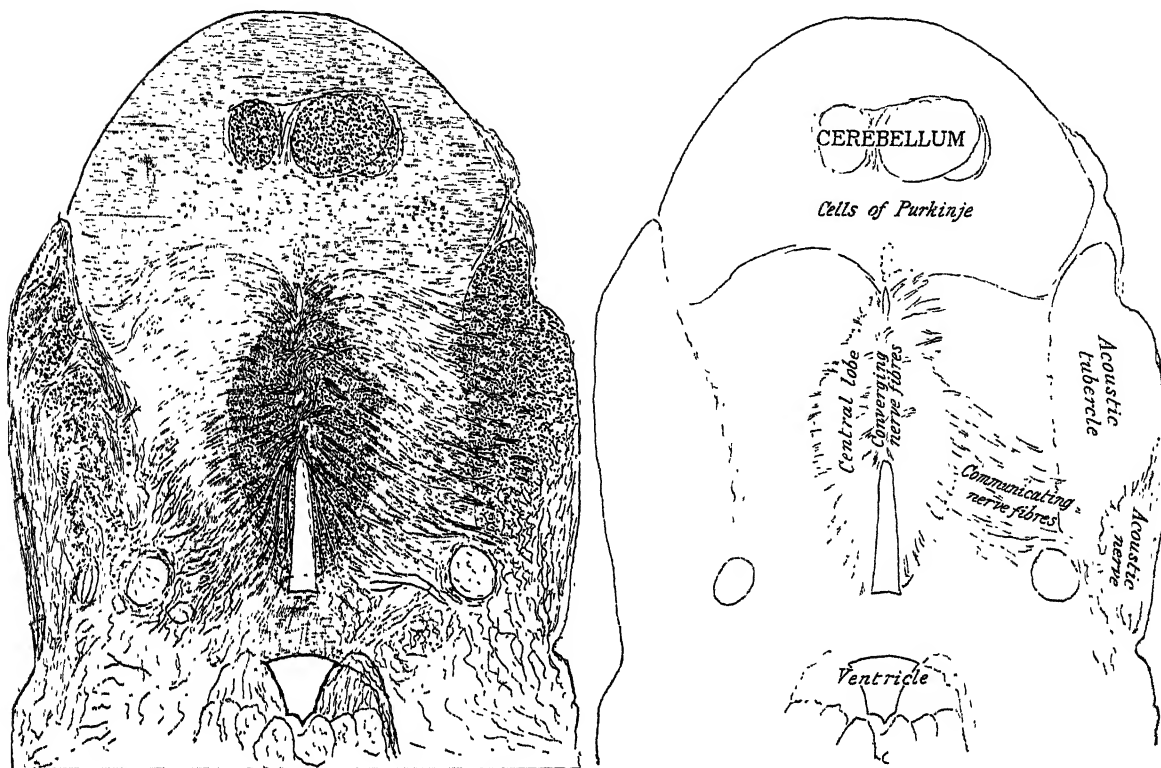
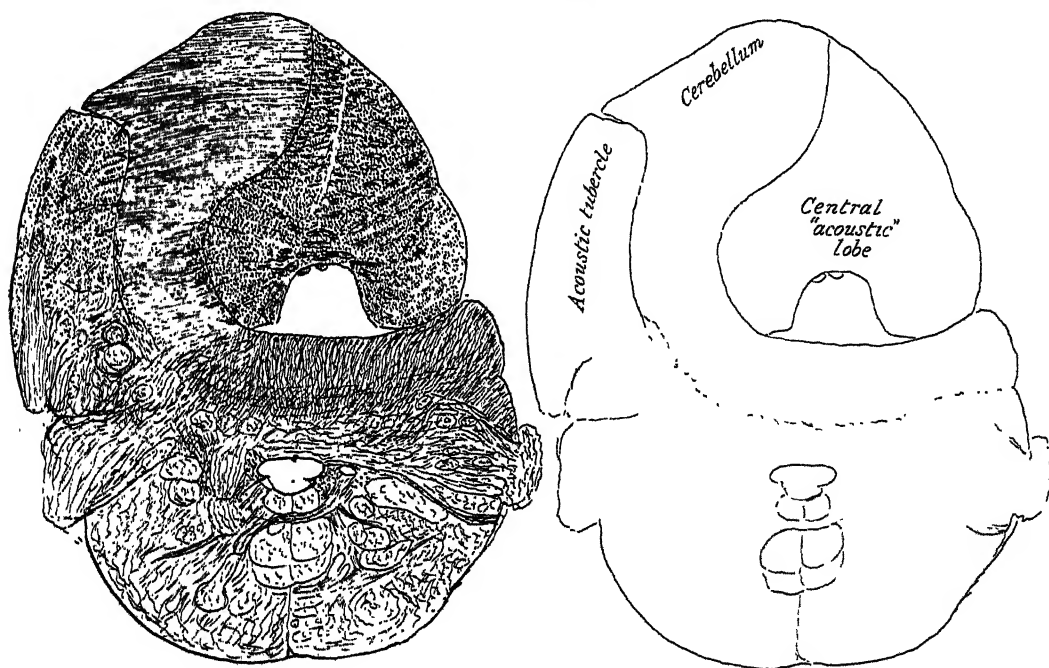


FIG. 19.—Herring.



and by its course, which seems always to be in a transverse direction leading straight to the ventricle. In the Herring it is clearly seen passing inwards, fig. 22, then marking a sharp turn caudally. Its fibres can be traced into a somewhat triangular mass of tissue lying on either side of the ventricle and from this tissue more posteriorly, fig. 23, fibres are seen to pass downwards into the great longitudinal secondary gustatory tracts, just as is found in the Cyprinidæ. The result of this examination of the so-called facial lobe

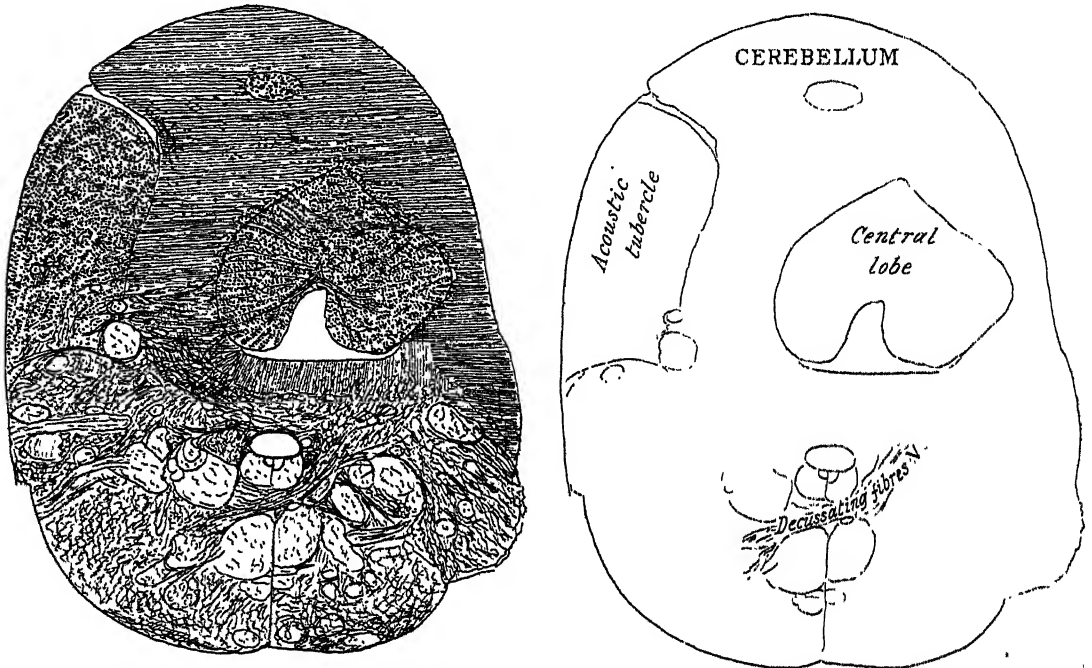


FIG. 21.—Sprat.

of the Herring is to establish the existence of a central lobe of peculiar structure which projects backwards from the base of the cerebellum; this lobe is clearly connected laterally with the acoustic tubercles and must be considered part of the acustico-lateralis centre. The facial lobes do not appear on the surface, but are covered by the Vth lobe, and they are somewhat feebly developed.

The Acoustic Tubercle in other Cyprinoids.

Fig. 16 gives in diagrammatic form the relative size of the acoustic tubercles and central acoustic area in the Tench, the Minnow, the Carp, the Roach, the Gudgeon and the Loach. In the Tench there is a narrow layer of cells at the

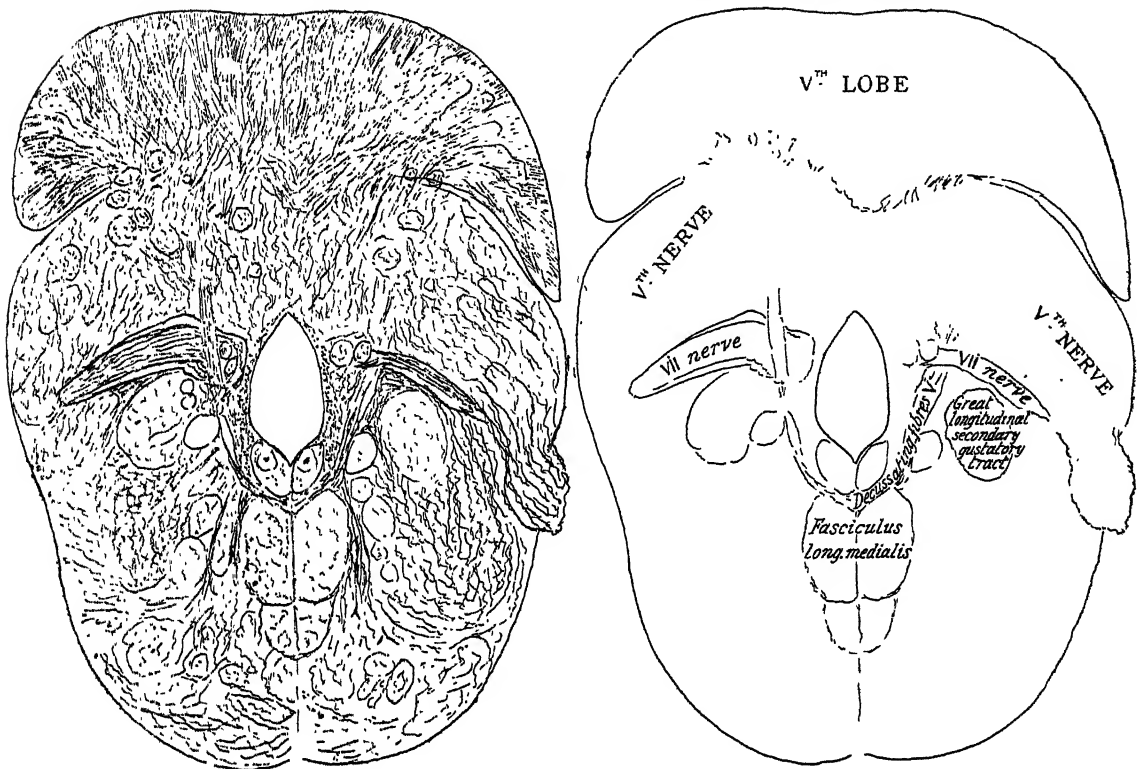


FIG. 22.—Herring.

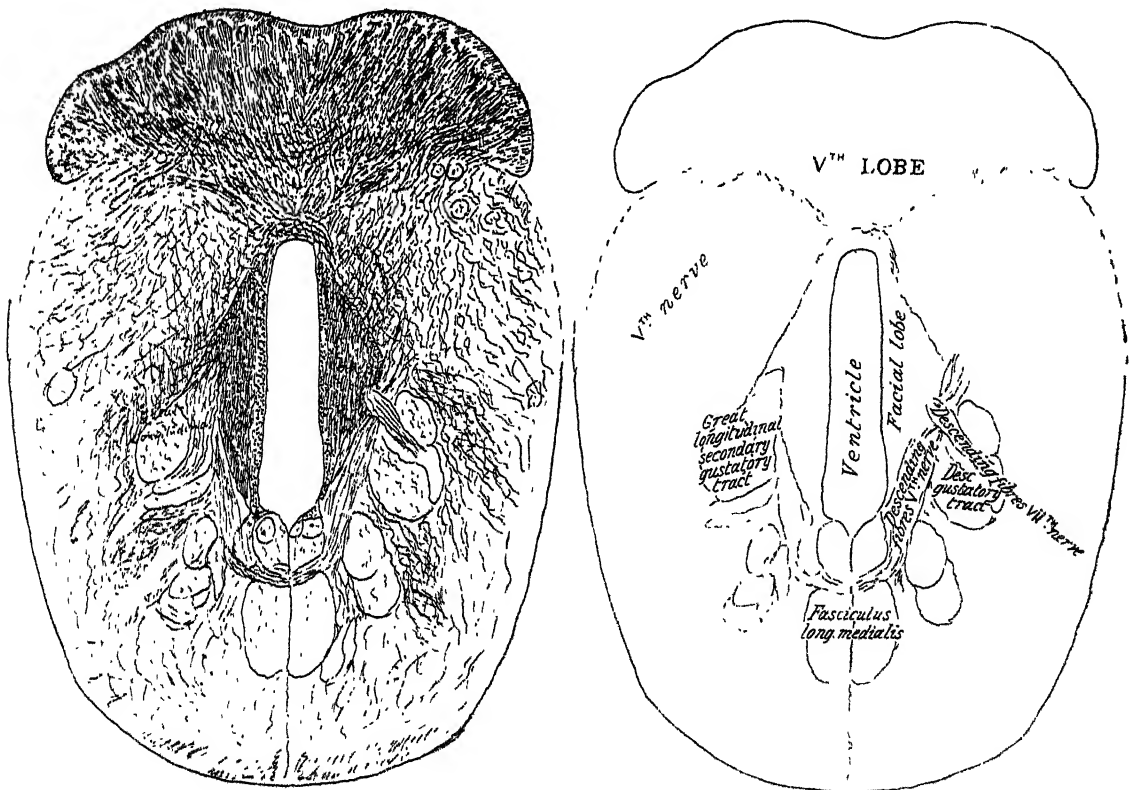


FIG. 23.—Herring.

base of the cerebellum, which diminishes in size as the sections are followed forwards; there is no central accumulation of round cells, but the acoustic tubercles are well developed.

The Minnow presents a somewhat similar picture, but the acoustic tubercles are less marked.

The Carp, the Roach and the Bleak, fig. 15, all present a well-marked central group of cells connected with the acoustic tubercles.

The Loach and Gudgeon show a narrow layer of cells extending across the base of the cerebellum, but this soon diminishes in size except at its lateral margins; there is no central accumulation of cells as in the preceding group of fishes.

We may therefore group the fish into three classes and for the sake of brevity call the central accumulation of round celled tissue the central acoustic area. In the purely bottom feeders, the Gudgeon and Loach, the central acoustic area is absent. In the fish which are both bottom feeders and frequently come to the surface for feeding or to suck air like the Roach, Carp and Bleak, the central acoustic area is well marked.

The Tench and Minnow form a third group in which there is a narrow layer of cells at the base of the cerebellum, but no definite central area. The Tench is essentially a bottom feeding fish, although on calm hot days it will come to the surface among weeds. As we have already noticed the Tench has a divided facial nerve and this feature is common to it, the Barbel, the Gudgeon and the Loach.

The Minnow prefers clear streams with a sandy or gravelly bottom, and in the larger rivers are often to be found where the water deepens at the end of a shallow (Regan, 1911).

They do not appear to have a great range in the depths in which they swim and in an aquarium are always seen to swim at a much lower level than the Roach or Goldfish.

In the light of the remarkable development of the acoustic lobe in the Clupeoids it would appear that this lobe is highly developed in those fish which have a wide range of movement at one time at the bottom and at another on the surface.

Both Cyprinidæ and Clupeidæ have a specialised connection of the swim-bladder with the auditory organ, and we find those Cyprinoids with a wide range of movement have a central acoustic area well developed and presenting some similarity with the acoustic lobe of Clupeoids.

The Acoustic Tubercle of the Whiting, Gadus Merlangus, and the Gurnard, Trigla Gurnardus.

We have examined the medulla oblongata of a physoclistous fish with a ground habitat to see whether a fish with a small range of movement as regards the depths at which it feeds would possess a central acoustic area. For this purpose we choose the medulla oblongata of the Whiting and the diagrams in fig. 15 show no trace of this area although the acoustic tubercles are large. This observation suggests that the central acoustic area may be connected with the control of the hydrostatic function of the swim-bladder. It is well known that the Gadidæ are readily distressed if pulled up from a depth owing to the slowness of the absorption of the expanded gases by the "oval" of the swim-bladder.

There would under natural conditions be no need for any great development of the centre controlling the alterations of the gaseous volume in the Whiting. But if this reasoning be correct, we should expect to find the same absence of a central acoustic area in other physoclists. We therefore examined the medulla oblongata of the Grey Gurnard, *Trigla gurnardus*, and we found contrary to our expectation that this fish has a very well-developed central acoustic area. This is shown in the drawing fig. 24.

The cerebellum is prominent and elongated in the dorso-ventral axis. A very well-marked central acoustic area appears in sections while the cerebellum still projects freely over the rhomboid fossa. This extends completely across the base of the cerebellum and is separated inferiorly from the lower margin by curiously interlacing nerve fibres. The stratum granulosum is large and has the shape in section of a cocked hat. As the central acoustic area is traced forwards the central portion gradually diminishes in size until only two small lateral areas are present at the level at which the acoustic tubercles become prominent.

Nerve fibres are seen connecting the tubercles and the small lateral portion of the acoustic area. The acoustic tubercles become very well developed.

If, as it is largely held, the Gurnards produce sounds by the contraction of the swim-bladder we should expect to find the acoustic tubercles well developed, and it is an interesting observation that in addition to their prominent tubercles there should be found this very highly developed central acoustic area; an area more extensive than in the Cyprinoids, but not forming a definite lobe as in the Clupeoids. It is certainly suggestive that, taking into account its purely ground habit comparable in a way with that of a Gudgeon or Loach, the presence

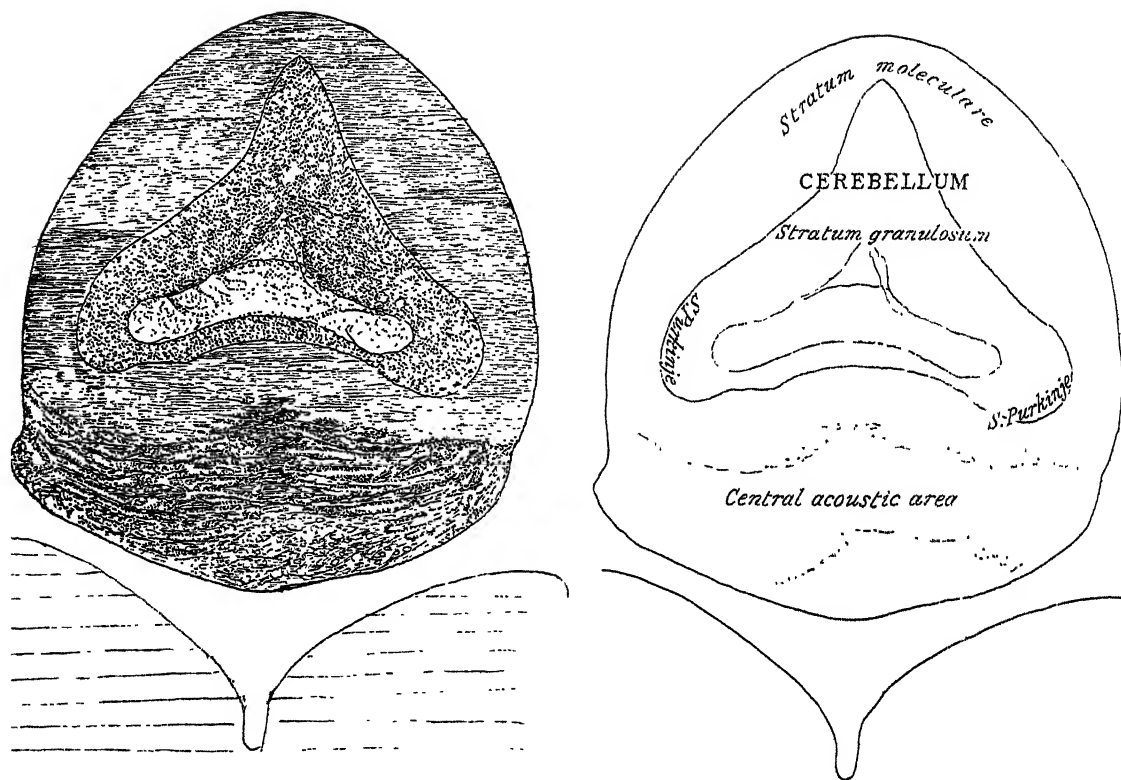


FIG. 24.—Grey Gurnard.

of this marked acoustic area in the Gurnard may be associated with an auditory function, especially when we recall the grunting powers of this fish.

Discussion.

The question of hearing in fishes has been discussed so often that as an introduction to any review of the auditory organs and their central nervous relationships it is well to state definitely what is meant by hearing in fishes. We are indebted to Parker (1918) for an exhaustive critical survey of the sense of hearing in fishes and we cannot do better than quote his definition.

“ The test for hearing in fishes is the proved presence of a response mediated by the ear and dependent on some vibratory physical disturbance in the water, which disturbance may vary from the extreme regularity of a pure tone to the extreme irregularity of a noise, such as the report of a gun or other like explosion.”

We would, however, suggest that Parker's words "mediated by the ear" as a test of hearing is an argument of questionable value, for obviously the primitive reaction of the organ we call the ear is to disturbances in the water; conceivably these at first do not excite a consciousness of hearing. We are all aware that vibrations of low frequency can be felt, and at the same time heard; we are also familiar with what is termed bone conduction, which can occur in the absence of the drum and ossicles of the middle ear, and it is obvious that in a fish, living in a fluid medium, vibrations conducted through the body walls would readily stimulate the sacculus and lagena.

We have seen that Clupeoids and Cyprinoids have a central "acoustic" area, which in the former is a definite lobe; and that among the Cyprinoids this area is more developed in the surface feeding species than in the ground feeders.

We propose to discuss the function of this specialised area and it seems that the method of comparative anatomy alone holds out any prospect of a correct solution of the problem. It would appear that there are three possible functions of this centre: (1) it may be a centre connected with the lateralis system; (2) it may be associated with the control of the buoyancy of the fish through the medium of the swim-bladder; or (3) it may have an auditory function. In this connection we will quote Herrick (1924): "the cerebellum develops from the forward end of the acustico-lateralis areas which in fishes are mixed auditory, vestibular and lateralis correlation centres." "The vestibular compound of the mixed acustico-lateral area is smaller than the lateralis and it is also intimately related with the cerebellum while the small auditory component is not known to have any cerebellar connections."

The first theory can be readily dismissed: when we consider that the Herring is characterised by the absence of a lateral line and that this is well developed in the Carps, it is hardly conceivable that the central acoustic lobe of the Herring can have reached its great development in relation to the lateralis system.

Certain facts support the hydrostatic theory. The Herring has a highly developed swim-bladder with a second pneumatic duct posteriorly, and the auditory connections of this organ with the ear are complex and unique. The Cyprinoids have a divided swim-bladder with a Weberian (Weber, 1820) mechanism connecting it with the ear; and in those Cyprinoids which both feed on the bottom and also frequent the upper planes of the water, the central acoustic area is well developed as in the Roach and Bleak, whereas in the purely ground feeders like the Gudgeon and the Loach this area is feebly developed.

When we examine the acoustic area of a fish like the Whiting, a type of the Gadidæ, with a closed swim-bladder that has no connection with the auditory organ we find that there is no central acoustic area connecting the acoustic tubercles. Moreover, since this fish feeds mostly at the bottom and exhibits great distress if it is rapidly hauled from a depth owing to its inability to adjust the distension of its swim-bladder, the absence of the central acoustic area is not surprising. Nevertheless if this argument is to hold good we should expect a fish like a Gurnard, also possessing a closed swim-bladder with no auditory connections and with a purely ground habit, would also be characterised by an absence of the central acoustic area. This is not so. Not only is the acoustic area well marked in the Gurnard, but it extends right across the medulla oblongata, almost meeting the enlarged acoustic tubercles on either side. This seems to put the theory of a hydrostatic function entirely out of court and we are led to the consideration of the auditory theory.

We can best consider this first of all in the light of what is known about hearing in the Gadidæ, Cyprinidæ, Clupeidæ and Triglidæ. As regards Gadidæ we know little if anything, but we must observe that their habitat is mostly at considerable depths. There are a number of observations on the hearing of Cyprinidæ, and it is now more generally held in the light of recent work that the Weberian ossicles have some auditory function. It is now known that the tripus is held in position by a special muscle and that the chain of ossicles can vibrate as the result of impulses conducted to the tense anterior sac of the swim-bladder. It is also known from the work of Bridge and Haddon (1893) that the sensory epithelium of the two saccules alone receives stimuli from the Weberian ossicles.

As regards the Herring and the Pilchard, Couch (1867) describes how they are readily frightened even by distant noises; it is also a custom among fishermen at Whitby and in Cornwall respectively to tap the bottom of their boats to make the Herring or the Pilchard swim.*

These observations cannot be lightly disregarded. We now come to the Triglidæ, well known for the grunting sounds produced by their swim-bladder. It is hard to believe that a fish that can produce grunting sounds is unable to receive auditory vibrations, so we may assume that we have evidence that the Gurnards possess some power of hearing. If our contentions are correct, there is definite evidence that these three families can and do hear. If therefore the acoustic lobe and area are developed in connection with the power of hear-

* Note on a method of fishing for Herring at Scarborough, see Appendix.

ing, we can readily understand how it is that the Herring has a well-marked central acoustic lobe because in this fish, as has been noted before, there is a complex connection by means of the spherical air vesicles between the swim-bladder and the ear, which is probably associated with audition.

Again, we have seen that those Cyprinidæ which come frequently to the surface to feed have a more marked acoustic area than the purely ground feeders; the latter would have little use for an organ of hearing. The noise-producing organ of a Gurnard would surely require the presence of a well-developed central acoustic area to enable it to appreciate sound, and in this connection we may quote the following (Parker, 1918):—

“Certain Teleostean families like the Siluridæ, Sciaenidæ and Triglidæ seem distinguished above all others by the prevalence of some form of vocal organ” and use the air-bladder as a sound-producing organ. The Sciaenidæ is a large family with about 150 species. The “maigre” or meagre (*Sciaena aquila*) is common in the Mediterranean and is sometimes taken on our coasts.

H. M. Smith, in 1905, made observations on the drumming among Sciaenidæ. He found that in those fish that drum the otoliths of the sacculi were exceptionally large, whereas in *Menticirrhus*, a sciaenid which does not drum, they are relatively small.

Parker, in 1903, described the deep drumming sound produced by the squeteague, *Cynoscion*, which is audible when the fish is in the air at a distance of 50 feet. This sound is produced only by the males, and it results from vibrations produced by a special muscle on the abdominal organs and particularly on the swim-bladder. The females not only do not drum, but possess no special muscle. It must be allowed that unisexual sound production strongly suggests the power of hearing.

We have, however, not had an opportunity of examining sections of the medullæ oblongatæ of Siluridæ or Sciaenidæ.

In conclusion we would suggest that the evidence so far as it goes is strongly in favour of an auditory function for the central acoustic area or lobe.

Summary.

(1) The form of the medulla oblongata of a plankton-feeding Cyprinoid is distinctive; its chief characteristic being a very small facial lobe which does not appear superficially as in other Cyprinoids.

(2) The Herring, a typical plankton-feeder, has a central lobe (wrongly called facial) composed of groups of round cells separated by strands of nerve fibres which join up with the acoustic tubercles. We suggest that this should be called the *central acoustic lobe*.

(3) There is a central area of similar cells and nerve fibres in the same position at the base of the cerebellum in Cyprinoids which, however, does not project into the rhomboid fossa. This area is also connected with the acoustic tubercles and we suggest for it the term central acoustic area. It is most marked in those Cyprinoids which feed on the surface.

(4) The central acoustic lobe and central acoustic area are found in those families that have a highly specialised connection of the swim-bladder with the auditory organ, the Clupeoids by means of the spherical air vesicles and the Cyprinoids by the Weberian ossicles.

(5) A central acoustic area is absent in the Whiting, a type of the Gadidæ with a closed swim-bladder.

(6) A central acoustic area is present in Triglidæ with a closed swim-bladder. This family produces sounds by the contraction of its swim-bladder.

(7) A review of the conditions under which the acoustic area and lobe are found leads to the conclusion that their function is probably auditory.

I wish to thank Mr. B. G. Clarke for his assistance in the preparation of the sections, and Mr. Michael Graham for the specimen of *Engraulicypris* which has proved so helpful in this research.

APPENDIX.

The Auditory Organ of the Herring.

The auditory organ of the Herring has been described by Weber (1820), Retzius (1884), Matthews (1886) and Ridewood (1892).

The description given by Retzius of utricle, sacculus and lagena corresponds to that of a typical fish with its anterior and posterior vertical semicircular canals and an external horizontal canal.

The Herring, like other Clupeoids, has a further system of bony cavities which are known as (i) the pear-shaped air vesicles into which posteriorly enter the ducts resulting from a division of the duct leading from the swim-bladder, and (ii) the anterior, and (iii) the posterior spherical air vesicles which communicate by narrow ducts with the pear-shaped vesicle. In addition there are certain canals and spaces which are in connection with the vestibule

but separated from it by a membrane from its main cavity, and these contain acoustic papillæ and lead to spaces lying immediately beneath the cutaneous covering of the head. According to the Cambridge Natural History (1904) "the tubular prolongation from the air-bladder, leading into the periotic vacuities, is open and the sac-like ends are in actual contact with protruding outgrowths from the utriculus."

Gunther (1880) describes the condition in Clupeoids as follows: "the narrow anterior end of the air-bladder is produced into a canal at the base of the skull and is divided into two very narrow branches which again bifurcate and terminate in a globular swelling. An appendix of the vestibulum meets the anterior of these swellings and comes in close contact with it. Besides the two vestibules communicate with each other by a transverse canal crossing the cranial cavity below the brain."

Some important details in the connection of the anterior spherical air vesicle with the vestibule may now be mentioned.

The examination of serial sections of the cranial cavity and brain of a young Herring cut in a horizontal longitudinal plane enables one to picture in detail a remarkable structure which enables any alteration of pressure in the swim-bladder to be communicated to the vestibule.

Fig. 25 is a section across the inferior portion of the auditory cavity. The sacculi are shown with the otoliths and nerve endings. In the outer wall of the sacculus is seen the pear-shaped air vesicle communicating anteriorly with the anterior spherical air vesicle which is divided into two segments by a transverse membrane which runs obliquely across the vesicle.

The duct enters the outer segment, while the inner division is small. This gradually diminishes in size if sections be followed ventrally. Posteriorly on the right the pear-shaped vesicle is seen to join up with the fine duct, the result of the division of the anterior prolongation of the swim-bladder.

Fig. 26 is a section at a more dorsal level than fig. 25. It cuts across both air vesicles and a portion of the pear-shaped vesicle which communicates with the posterior air vesicle. A fine membrane is seen detached in the posterior and pear-shaped vesicles which is continuous with the lining of the duct from the swim-bladder. In the anterior air vesicle there is also to be seen a detached membrane which, however, only lines the outer division of the air vesicle which we have already seen is in communication with the swim-bladder. Under the epidermic covering of the head is a lateral canal with an end-organ surmounted by a small otolith; this is protected by a sickle-shaped ossicle with its shaft prolonged posteriorly, an enlarged view of which is shown in fig. 29.

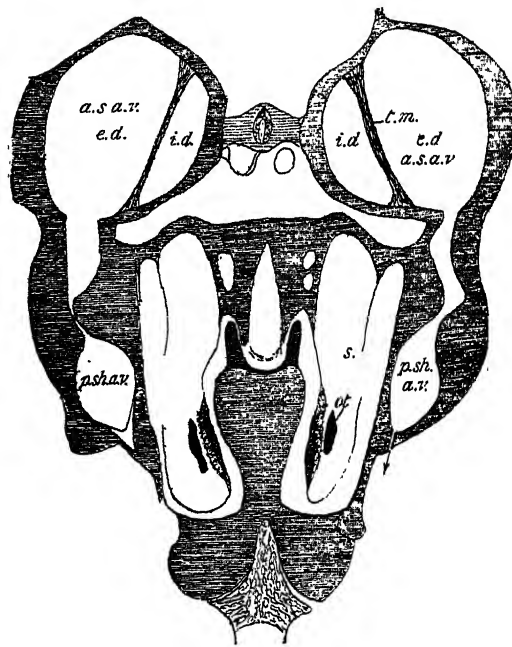


FIG. 25.—*i.d.*, internal, *e.d.*, external division of anterior spherical air vesicle; *p.sh.a.v.*, pear-shaped air vesicle communicating at ↓ with duct from swim-bladder; *t.m.*, transverse membrane; *s.*, sacculus; *ot.*, otolith; *a.s.a.v.*, anterior spherical air vesicle.

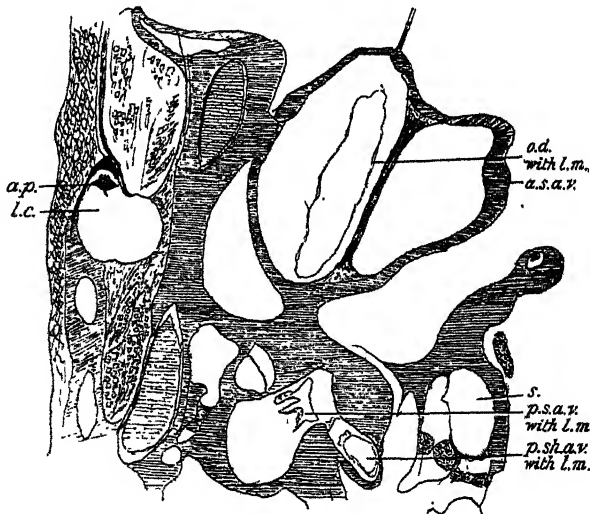


FIG. 26.—*o.d.*, outer division of anterior air vesicle; *p.s.a.v.*, posterior spherical air vesicle; *s.*, sacculus; *l.c.*, lateral canal; *a.p.*, acoustic papilla.

Fig. 27 is taken from a section still more dorsal. Both air vesicles are seen, the posterior no longer shows its communication with the pear-shaped vesicle. The anterior air vesicle has undergone a great change (in section). The internal division is now considerably larger than the external which still shows the detached lining membrane. There is now an opening in the posterior wall of the internal division, leading into the vestibule, and attached by a fibrous band to the inner lip of this orifice is an ovoid piece of cartilage. This process bears on its free extremity a layer of cells with fine hairs and it is to be noted that there is no otolith lying on their margin; the process swings like a valve

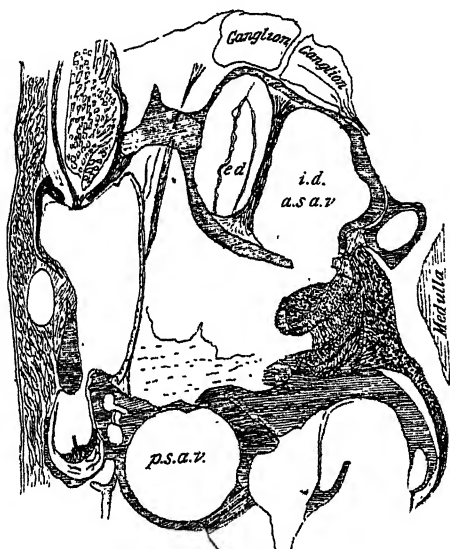


FIG. 27.

across the orifice, and is connected by fine nerve fibres with a large ganglion which joins the auditory nerve anteriorly at its junction with the medulla.

The lateral canal noted in the preceding section is no longer separated by a bony wall from the vestibule, but appears as an elongated space, the inner boundary of which is formed by a fibrous membrane.

Posteriorly a second papilla with otolith is seen which lies in a smaller canal joining the above-mentioned space. In the wall of the internal membrane nerve fibres are seen which pass to a ganglion lying against the anterior wall of the anterior spherical air vesicle.

Fig. 28, the last of this series of four sections, is still more dorsal and cuts across the anterior air vesicle above its opening into the vestibule; the external division is now very small. The posterior vesicle is now partially surrounded

by the horizontal semicircular canal, and portions of the acoustic papillæ of the anterior vertical and horizontal canals are shown. The elongated space of the last section is now very much prolonged posteriorly and two papillæ with otoliths are shown, the nerves from which pass up in the wall of the internal membrane and pass external to the anterior air vesicle to end in the ganglion on its anterior surface.

It will be seen from this description that any alteration in the pressure of the gases in the swim-bladder will affect the external division of the anterior

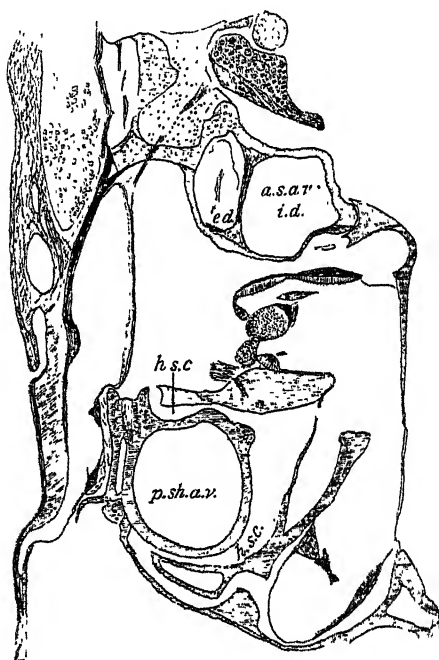


FIG. 28.

air vesicle. There will therefore be a movement communicated to the transverse membrane which will be transmitted to the fluid in the vestibule. If the volume of gas is increased in the air-bladder a flow of fluid will take place from the internal division through the vestibular orifice, this will impinge on the cartilaginous process lying against its entrance and cause an impulse to be received by the hair cells which would pass to the ganglion above described.

We must mention here the existence of a triangular shaped fenestra "in the side or rather in what from its form must be called the base of the Herring's skull. Its posterior end is bounded by the descending part of the exoccipital which contains the swim-bladder duct. This fenestra is surrounded by con-

nective tissue and muscle immediately dorsal to the pharyngo-brachials and is covered by a delicate tense semi-transparent membrane within which is closely applied the sacculus of the ear with its contained large otolith. This can be readily seen shining through the membrane which almost bears the appearance of a tympanum " (Matthews, 1886), figs. 30, 31, 32.

The presence of a fenestra in the wall of the sacculus closed by a membrane, suggestive of the membrana tympani of man, is an anatomical fact of considerable importance. When we consider that the membranous sac in the outer segment of the anterior air vesicle is in direct communication with the swim-bladder, and that it is only separated from the endolymph within the sacculus by the transverse membrane, fluid being incompressible, any increased

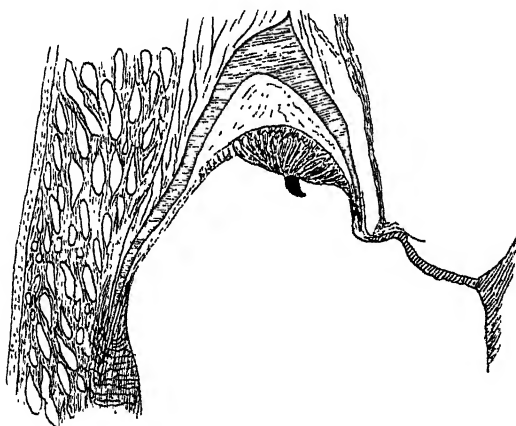


FIG. 29.

pressure on the transverse membrane would be conveyed to the membrane of the fenestra. This would tend to obstruct its response to a vibration received through the body walls. But when fluid is driven out of the internal segment of the anterior air vesicle it impinges on the hair cells that cover the margin of the valvular projection that overlaps the foramen.

We would suggest that this gives rise to a stimulus which is received by the acoustic centres, and that, as a result, impulses are sent thence to the swim-bladder which releases the pressure of the expanded gases by means of its posterior pneumatic duct; the pressure on the fenestral membrane is thus reduced, and vibrations would be received normally. Observations on the reaction of Herrings to loud noises prove that the immediate impulse is for the fish to dive. The Scarborough and Whitby fishermen employ a purely local method of fishing—"beating for herrings." In August the Herring are then

some 10 miles from the shore in 13 fathom of water. Late in the evening the fishermen put to sea and keeping a close watch they wait till they see the surface of the water alter in colour and character as the mass of herring gradually "swim" or rise to the surface. They at once steam or row into the middle of this area and shoot their nets. As they do so every bucket, tin can or shovel available is used to make a din by beating against the sides of the vessel, accompanied by shouts and yells. Be it noted that the vibrations from the screw and the disturbance of oars is not sufficient to frighten the fish. But the noise produced by "beating" is so great that the fish dive to the bottom and are then immeshed in the nets which extend some 7 fathom down below the 3 fathom level at which the net commences. The reaction of the fish to loud noise is to make them dive; if they did not dive they would swim safely above the net.

It appears that the power to perceive noise is, owing to the art of the fisherman, disadvantageous, and further if there were no mechanism to control the pressure in the swim-bladder, the fenestral membrane would be unable to vibrate and the fish would escape. Nature had not visualised that the danger area was beneath them; it would have been expected that the danger was on the surface whence the noise emanated.

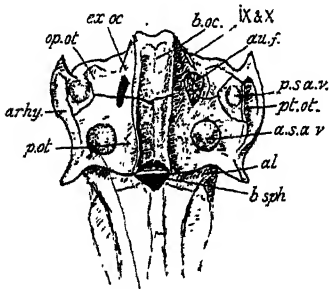


FIG. 30.

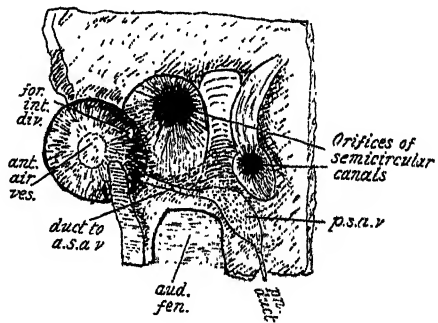


FIG. 31.

au. f., auditory fenestra; *a. s. a. v.*, anterior spherical air vesicle; *p. s. a. v.*, posterior spherical air vesicle.

Fig. 30 is redrawn from a figure in the Appendix to the Annual Report of the Scottish Fishery Board (Matthews, 1886). It represents the base of a Herring's skull and it shows the position of the anterior and posterior spherical air vesicles and the auditory fenestra.

Fig. 31 is an enlarged drawing to show relations of auditory fenestra.

Fig. 32 is a dissection of one-half of the skull of a Herring which has been divided through the anterior spherical air vesicle and exposes the auditory fenestra. A bristle has been passed through the external division of the vesicle and this can be seen passing through the pear-shaped vesicle and curving over

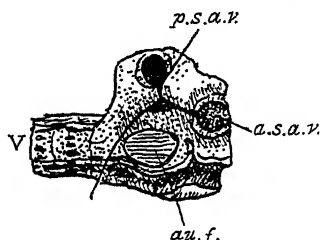


FIG. 32.—*v.*, vertebral column; *p.s.a.v.*, posterior spherical air vesicle; *a.s.a.v.*, anterior spherical air vesicle; *au.f.*, auditory fenestra.

the fenestra and making its exit posteriorly. The posterior air vesicle has been filled with Indian ink and the canal leading to it from the pear-shaped vesicle is also shown by the same method, so that their position is seen without removing their walls.

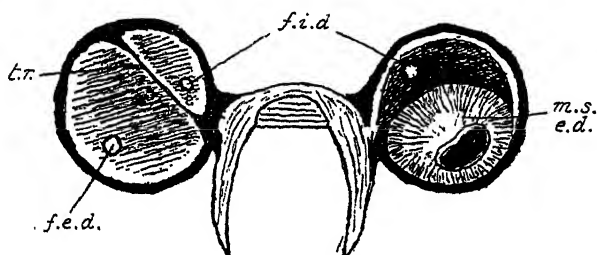


FIG. 33.—Herring. A semi-diagrammatic view of the anterior spherical air vesicles, the anterior walls of which have been removed. On the right the wall of the vesicle has been chipped away to expose the delicate sac *in situ*. A hole has been made in the wall. On the left the sac has been removed to show foramen. *f.e.d.*, *f.i.d.*, foramen of external and internal division; *m.s.e.d.*, membranous lining of external division; *tr.m.*, transverse membrane.

[*Note added in proof, July 12, 1932.*—Since writing this paper the medulla oblongata of the Pilchard has been examined. It is found that it presents a well-marked central acoustic area which may be described briefly as a transitional form between that of the Bleak and the Sprat.]

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Discussion on Recent Advances in the Study of Enzymes and their Action.

(June 16, 1932.)

THE PRESIDENT: We meet this afternoon to discuss Enzymes and their action. So much important work has been done on that subject during quite recent times that I think you will admit that the occasion is timely. The Royal Society is honoured to-day by the presence of distinguished guests from abroad. I would like to say how deeply we appreciate their presence. It is not proposed that our discussion shall follow pre-determined lines, but rather that each speaker should deal with any aspect of the subject with which his present interest is concerned. It is not my duty, therefore, to attempt from the Chair any preliminary survey of the field of discussion. In opening, I will be very brief. I wish to call your attention to certain observations made on bacteriological enzymes by my colleagues, Miss Margaret Stephenson and Dr. L. H. Stickland.

They have recently shown that the enzymes of *Bacillus Coli* activate hydrogen in three different senses or modes. They can activate molecular hydrogen when present in the atmosphere to which the organisms are exposed. As a result, reductions occur in the medium which are not observed in the

absence of the organism, nor when free hydrogen is absent from the system. My colleagues were the first to show that this activity is possessed by *B. Coli*, and no less by some other common bacteria.

Intramolecular hydrogen is also activated by *B. Coli*, and that in two modes leading to distinct results. The first of these is an instance of an ordinary dehydrogenase effect: an organic molecule is so activated that it becomes a Donator of hydrogen which is then transferred to an Acceptor. In the second case free molecular hydrogen is liberated from an activated molecule. As an example of this last, Stephenson and Stickland have studied the breakdown of formic acid into hydrogen and carbon dioxide. Thus the following types of reaction are controlled by the organism :—

- (1) $H_2 + A \rightarrow AH_2$ (Hydrogenase)
- (2) $H_2D + A \rightarrow AH_2 + D$ (Dehydrogenase)
- $H.CO_2H + A \rightarrow AH_2 + CO_2$ „
- (3) $H.CO_2H \rightarrow H_2 + CO_2$ (Hydrogenlyase)

It is a point of interest that the authors have clearly shown that the catalyst is distinct in each of the three cases mentioned. They propose the above nomenclature for the three enzymes concerned. We have here an illustration of high specificity in enzyme action. There are some, it would appear, who feel intellectual difficulty in admitting that this apparent high specificity among intracellular enzymes can be real; perhaps because it implies so great a complexity in the equipment of the living cell. I am bound to say that I do not share that feeling. When I try to understand the extraordinary degree of organisation of chemical events which obtains in living systems, I am myself much aided by a realisation of the organising potentialities inherent in highly specific catalysis; whereas without that aid I can obtain no understanding at all.

I wish to speak particularly of the third type of reaction discussed above.

If *B. Coli* be grown in a broth medium containing formic acid, there is a breakdown on the lines of this type. If a culture so grown be washed and suspended in a medium which contains formates, but which does not allow of growth, the quiescent organisms still actively catalyse the same reaction. If, on the other hand, they are first grown on the broth medium without formates, they entirely fail to show this activity when afterwards washed and suspended. During the plastic period of growth, therefore, contact with the formic acid molecule leaves some impress upon the structure of the cell, which endows it with a new catalytic agency. With this technique there can

of course be no question of any selective survival such as is sometimes thought to explain the apparent adjustment of cultures to unaccustomed substrates. This observation is not unique, though a particularly clear instance of the phenomenon in question. Similar behaviour has been recently shown, for example, to be displayed by *B. aerogenes* in relation to the fermentation of xylose (Karstrom).

We must doubtless be cautious in the interpretation of such results, but it does seem to me that they are so clear and so definite that an explanation of them ought in the future to be reached on experimental lines. At any rate, if in such experiments we really observe the birth of new enzymes, then I think an account of them forms a suitable opening for this discussion.

Professor R. WILLSTÄTTER : Among recent advances in the study of enzymes there are in particular four lines of research which I should like to consider. First of all, there is the knowledge of the limits of activity by which the enzymes in most cases are solely characterised, at the start and for a long period, for this is the only way of defining them as long as nothing can be said concerning their chemical composition. It is to be remembered that the constitution of an enzyme cannot be determined in the same way as the structure of simpler substances such as hormones and vitamins, which may now be so considered. We form a preliminary and approximate image by assuming them to be built up of a specifically active group and of a colloidal carrier. Concerning the structure of both parts, mention must be made of new methods of analysis and realisation. There is another question closely connected with the nature of the colloidal carrier. It is the problem of the binding of the enzymes to the cellular substance—to the plasma—and the processes of their dissolution out of the cells. Finally, we are going to mention briefly some new ideas on the nature of enzymatic action.

Specificity of enzymes cannot be determined exactly as long as one enzyme is mixed with others of the same group ; for instance, one proteinase with another, a peptidase with several other peptidases, the carbohydrases splitting, be it saccharose, be it maltose with one another. The pancreas, for instance, secretes a mixture which consists of numerous components. The separation of such natural mixtures by adsorption methods is nowadays a necessary and essential means for the determination of the range of reaction of each enzymatic component. Waldschmidt-Leitz and Grassmann have separated into their components pancreatic proteases and other proteolysing mixtures of animal and plant origin. Of the animal proteases we know to-day the proteinases,

pepsin, trypsin, and kathepsin, amino-polypeptidase and carboxy-polypeptidase, di-peptidase and prolinase. As some of the names indicate, we can determine which atomic group of the substrate, the amino group or the carboxyl end, forms the point of attack. A research of Grassmann in Munich, not yet published, completes our knowledge of the carbohydrases and corrects the views of Weidenhagen concerning their specificity. Enzyme mixtures from *Aspergillus* contain on the one hand a β -gluco-poly-saccharase, splitting cellulose, lichenin, cellodextrine, and, on the other a β -gluco-oligo-saccharase, which hydrolyses cellobiose, triose, tetraose (and β -glucosides). The behaviour of both of them is identical towards many adsorbents, but by means of one certain alumina (AlO_2H) they can be separated quantitatively, since polysaccharase is not adsorbed. This is the same alumina by which a few years ago saccharase and maltase were separated. Also for a few enzymes the specifically active groups have recently been recognized on the basis of their spectroscopic properties, and this should lead to important progress in analysis. This discovery concerns enzymes which are closely related to each other, in particular the respiratory ferments of Keilin and Warburg, and the catalase of Teile and the peroxydase of Kuhn. They contain iron bound to porphyrines, but the colloidal carriers have not yet been determined. Thus indeed we are very near to setting up a chemical formula for an enzyme, not very differently from what has been attained for chlorophyll, at least a formula for the active group. With regard to the nature of the colloidal carrier of an enzyme, hotly contested advances are being made. As many enzymes are observed only in a very impure state, accompanied by the most diverse colloidal substances, one can to-day attempt to throw light upon the nature of the carrier only by determining which colloids can be dispensed with. But there is the danger that the chemical reactions proving the presence of proteins and carbohydrates are not sufficiently sensitive, when the enzyme preparations are obtained in a condition of high activity. The Millon test and the tryptophane reaction came out very strongly in the case of ordinary preparations of saccharase, but with newer methods of isolation and purification these protein reactions entirely disappeared. Sherman found protein to be an essential constituent of pancreatic amylase; on the other hand, Waldschmidt-Leitz could free this enzyme completely from protein by a process of precipitation with acetone. The researches of Sumner and of Northrop have an important bearing on the question whether protein is really a necessary constituent of the enzymes. While the Munich laboratory endeavoured to purify enzymes so that they should be more and more free from protein, Sumner and Northrop,

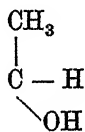
by contrast, have employed the methods for the crystallisation of proteins to produce enzymatically active preparations in the form of proteins. Sumner succeeded in precipitating urease as a crystallised globulin, Northrop obtained pepsin, and, later on, also trypsin as crystallised proteins. Certainly these are beautiful preparations of enzymes. However, Waldschmidt-Leitz could decompose urease by proteolysis without loss of activity. And, as far as pepsin is concerned, earlier investigators—Brücke in Vienna, as long ago as 1861, and others—have described strongly active preparations which seemed to be obtained free from proteins, and such results can be corroborated. An explanation of these differences may be that pepsin itself, be it a protein or not, is diluted by foreign protein in the Northrop preparation, but in other preparations, for instance that of Brücke, diluted with a different type of ballast.

The composition of enzyme preparations, chiefly of the colloidal carriers, varies greatly because the autolytic processes, proteolytic or amylolytic reactions, by which the enzymes are brought into solution, are of manifold and diverse kinds. If we improve the methods of dissolution so as to exclude autolytic reactions, we frequently find that enzymes which hitherto had only been observed in soluble form are only partly soluble or in other cases entirely insoluble. We can now extend the field of enzyme research by studying the insoluble enzymes, bound to plasma, and by following their passage into solution. We find, for instance, that an essential part of pepsin is contained in insoluble form in the mucous membrane of the stomach and produces solutions of pepsin only under conditions of proteolysis. Of the intracellularly arising enzymes we propose to call those endo-enzymes which are insoluble owing to this inclusion in, and adsorption to the cellular structure, and are made free and become soluble by its destruction, even if it be only mechanical destruction. Such is the case with the carbohydrases of yeast, saccharase and maltase. From this we must distinguish the occurrence of enzymes bound chemically to protoplasm. These we call desmo-enzymes. They are insoluble owing to the chemical structure of the complexes to which they are anchored. The dissolution of a desmo-enzyme consists in the splitting off in the hydrolysis, of the enzyme complex itself. In the mucous membrane of the stomach we find desmo-pepsin besides soluble pepsin (lyo-pepsin), and in the pancreas, apart from soluble trypsin, desmo-trypsin, which can be obtained in an active as well as in an inactive form. Recently the desmo-enzymes of the leucocytes, for instance, desmo-trypsin, -amylase, -maltase have been observed and studied in greater detail. Several such desmo-enzymes show sharp differences from the lyo-enzymes. Desmo-amylases, for example, have

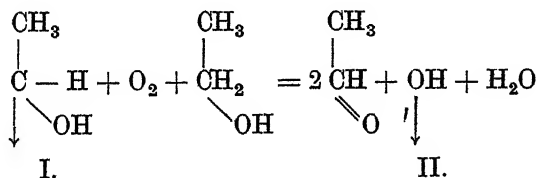
the characteristic of complete inhibition by glycerin but, on the other hand, they do not require the addition of phosphate, while the soluble amylase (α -lyo-amylase) of the leucocytes becomes active only through the addition of phosphate.

Enzymes become free and soluble by autolysis either in physiological processes or *post-mortem*. The same phenomenon may occur frequently with other physiologically active substances, which are also bound to the plasma, and become soluble by hydrolysis. We may therefore ask, in what state does, for instance, thyroxin occur in the gland? Free and soluble or rather insoluble, bound to a plasma complex?

The enzymes are colloidal products of high molecular weight. However, important characteristics can be demonstrated on simple models, for instance, according to Kuhn, on Fe-ion adsorbed to graphite. New knowledge regarding simple catalytic reactions can be applied to reactions of ferments. The ideas on chain reactions, developed by Haber and Franck for the autoxydation of sulphite, can be carried over to biological oxydations, dehydrogenations and dismutations. This hypothesis of Haber differs from earlier explanations in two respects. The assumption that hydrogen atoms are split off in pairs from organic compounds is replaced by the hypothesis of monovalent reactions; for instance, the splitting off of a single hydrogen atom is the first step of an oxydation giving rise to a radical. It had been assumed that under the action of the catalyst one mol of the reaction product would result, and that the unchanged or regenerated catalyst would then form a second mol of the product, and so on. The principle of the chain reaction should, on the contrary, consist in the production of a second radical from the first; for instance, dehydrogenated alcohol, the first radical,



should produce a second radical, that is hydroxyl,



and this again should generate the first one, and so on, so that one initial

impulse given by the catalyst causes the formation of thousands of molecules of the end-product.

DR. E. WALDSCHMIDT-LEITZ: The possibility that a chemical combination between enzyme and substrate led to the phenomenon of enzymatic specificity, was first suggested by Emil Fischer. He illustrated this view with the well-known analogy of lock and key, which carries with it the assumption that the compound formation is dependent not only upon chemical, but upon stereochemical relationships.

The enzyme reaction, therefore, takes place through an intermediary enzyme-substrate compound. There are to be distinguished, according to Michaelis, two different steps in the reaction, the formation and the decomposition of the enzyme-substrate compound, into free enzyme and the split-products of the substrate, which may be analytically differentiated. According to the so-called "Two-Affinities-Theory" of v. Euler, these two steps require two different active groups of the enzyme molecule, a substrate-binding and a substrate-splitting group. These correspond to two different enzyme-reacting groups in the substrate. The splitting of the substrate therefore takes place at some point other than that to which the enzyme is attached. This theory of enzyme-action thus resembles to a remarkable degree the "Side-Chain-Theory" of Paul Ehrlich, with its distinction between haptophore and functional groups. As a matter of fact, the nature of the groups both in substrate and in enzyme, which take part in some examples of peptide hydrolysis, can now be described. The considerations leading to these results I would like to tell you in detail.

The proteolytic enzymes are divided, according to their specificity, into the two large groups of proteinases and peptidases. The subdivision of the peptidases, like that of the proteinases, rests upon differences in their mode of attacking the peptide molecules. They are divided into carboxy-polypeptidases, amino-polypeptidases, dipeptidases, and imino-peptidases, according to the structural characteristics of the peptides or peptide derivatives, which are necessary for the attack of the ferment.

In nature the above enzymes occur only in mixtures in which the effects of the single enzymes cannot be recognized and differentiated. The isolation of these different types of peptidases, and the possibility of distinguishing between them, is due to the application of new methods of preparation. Most important among these methods is the process of adsorption and elution, introduced into enzyme-chemistry by Willstätter.

The investigation of the specificity of the peptidases has been of special significance. For the experiments with synthetic peptides and peptide-derivatives have increased our knowledge of the mechanism of proteolytic processes, and of enzyme reactions in general. Whether a peptide is split by the amino-polypeptidase or by the dipeptidase, for instance, is determined by the length of the peptide chain, while in the case of the carboxy-polypeptidase it is also dependent upon the nature of the component amino acids.

As is shown by the Table, the dipeptides are for the most part specific substrates of the dipeptidase. They are not attacked by amino-polypeptidase in any case, and only by carboxy-polypeptidase in a few instances where the peptide is characterised by a particular amino-acid content. The special influence of certain amino-acids is clearly seen by comparing the specificity of the two polypeptidases towards tyrosin-free and tyrosin-containing polypeptides. An increase in the length of peptides like glycyl-glycine and leucyl-glycine, brought about by merely adding glycine residues to the molecule, does not cause the peptides to react with carboxy-polypeptidase. The introduction of a tyrosine-residue, on the other hand, does, for, as the Table shows, the tripeptide leucyl-glycyl-tyrosine, the tetra-peptide leucyl-diglycyl-tyrosine and the pentapeptide leucyl-triglycyl-tyrosine are split by carboxy-polypeptidase. Of these, the tyrosine-containing pentapeptide furnishes a really specific substrate for the ferment, since it is no longer attacked by the amino-polypeptidase, though the tri- and tetrapeptides are. The modes of action of the two enzymes, however, are not the same. It may be shown that the action of the carboxy-polypeptidase involves the carboxyl end of the molecule, and stops with the splitting off of the tyrosine, while the hydrolysis through amino-polypeptidase takes place at the end containing the free amino-group.

These results naturally bring up the question of the deeper causes of the differences in specificity between the peptidases. To-day this question is on the way to being answered, thanks to the work done on the specific liability of peptide-derivatives. There are distinct structural requirements for the hydrolysis of a peptide by means of any particular enzyme. These structural features of the peptide molecule are necessary for the action of the enzyme, because through them the combination between enzyme and substrate takes place.

It has been shown that the dipeptidase and the amino-polypeptidase attach themselves to the free amino-group of the peptides. Naturally, then, for these enzymes, the presence of a free amino-group in the substrate, is a necessity. If it be altered, for instance, by the introduction of an acyl group, the enzymes

can no longer function. The free carboxyl-group of the peptide, however, may be removed without stopping the enzyme action. The effect of the amino-polypeptidase and of the dipeptidase, therefore, consists in splitting off the amino-acid which originally carried the free amino-group.

The union between the carboxy-polypeptidase and the peptides is of quite another character. This enzyme unites with the carboxyl-group, and its action consists in splitting off the amino-acid carrying the free carboxyl-group of the peptide. The difference between the two types of enzyme-peptide combination, characteristic of the two polypeptidases, is most strikingly illustrated by the fact that compound-formation with the amino-polypeptidase is affected only by the steric configuration of the amino-end of the molecule, while for compound-formation with the carboxy-polypeptidase only the steric configuration of the carboxyl-end comes into question. Accordingly, the hydrolysis of the racemic tripeptide *d*, *l*-leucyl-glycyl-*l*-tyrosine is asymmetric with the amino-polypeptidase, and is limited to splitting off the *l*-leucine, but with carboxy-polypeptidase the hydrolysis is symmetrical, leading to the formation of *l*-tyrosine and *d*, *l*-leucyl-glycine.

The enzyme-binding groups in the peptides are, then, for the dipeptidase and the amino-polypeptidase the NH_2 -group, and for the carboxy-polypeptidase the COOH -group. What, however, is the nature of the corresponding active groups of the enzyme molecule? Some light has been shed on this subject by investigating the variation of the enzyme action with the p_{H} , in the case of the dipeptidase. For the form of the p_{H} -curve is the same for the reaction of dipeptide with dipeptidase and for the condensation-velocity of dipeptide with glucose as well. From these observations the conclusion is justified that in the reactions dipeptide-glucose and dipeptide-enzyme, similar chemically active groups take part. As v. Euler and Josephson first suggested on the basis of inhibition experiments, the haptophore group of the dipeptidase which takes part in the anchoring of the substrate, and also probably of the amino-polypeptidase, might be considered to be an aldehyde or keto group. The anchorage of the peptidases on the peptide- NH_2 -group may quite well occur through the formation of a Schiff's base.

Our information about the haptophore group of the carboxy-polypeptidase is still less definite. The anchorage of the enzyme on the carboxyl-group seems to require a certain degree of ionization of the COOH . The previously mentioned influence of special amino-acids like tyrosine on the liability of peptides to splitting by carboxy-polypeptidase seems referable to the electrochemical nature of the carboxyl-group. The reaction of the carboxy-polypeptidase

with its substrates appears, therefore, to be an ionic reaction, and the haptophore group of the enzyme of basic nature.

The union of the ferment and substrate, that is, the formation of the enzyme-substrate compound, does not complete the enzymatic catalysis. It must be supplemented by the decomposition of the enzyme-substrate complex into free enzyme and the split-products of the substrate. There are different ideas concerning the mechanism of this decomposition.

Among the explanations, to-day at least, the most defensible makes the assumption of more than one union between the enzyme and the substrate and so corresponds to the "Two-Affinities-Theory." In the case of the dipeptidase, experiments have shown that the specific action of the enzyme is dependent not only upon the optical configuration of the amino-acid carrying the free amino-group, where the enzyme is known to attach itself, but also upon the optical configuration of the amino-acid carrying the carboxyl-group. For instance, in the racemic glycyl-*d*, *l*-alanine, only the naturally occurring glycyl-*d*-alanine is hydrolysed. There probably exists an atomic grouping in the peptide molecule which acts as a second-linking-point with the enzyme, and which belongs to the amino-acid residue carrying the carboxyl-group. According to Balls and Köhler, this second point of attachment, not only for the dipeptidase, but also for the amino- and carboxy-polypeptidases, is the imino-group of the peptide-linkage which is hydrolysed. This conclusion is reached by consideration of the hydrolysis of a series of substituted glycyl-anilines like the glycyl-amino-benzoic acids, and is corroborated by the behaviour of other substituted acid-amides as inhibitory substances.

All the peptidases, then, have this in common, that they unite with the imino-group of the peptide bond which is split. They differ, however, according to the other group in the substrate with which they first combine.

This assumption of the special function of the imino group in the decompositions of the enzyme-peptide compound is also corroborated by the greatly increased instability shown by peptides in which the imino-hydrogen has been substituted by an acid radical. If, according to Bergmann, the imino-hydrogen of a dipeptide is acetylated, the resulting substance decomposes spontaneously in weakly alkaline aqueous solutions, into its constituent amino-acids.

By these experiments on the mechanism of peptidase action the "Two-Affinities-Theory" has shown itself to be a valuable working hypothesis. It permits the explanation of enzyme action as an ordinary chemical reaction between definite chemical groups in enzyme and substrate, the variation in

whose chemical or electrochemical affinities accounts for the phenomenon of enzymatic specificity.

TABLE.—Specific Splitting of Peptides.

Peptide.	Dipeptidase.	Amino-poly-peptidase.	Carboxypoly-peptidase.
Dipeptides—			
Glycyl-glycine.....	+	—	—
Leucyl-glycine.....	+	—	—
Glycyl-tyrosine	+	—	—
Histidyl-glycine	+	—	—
Phenylalanyl-arginine	+	—	+
Glutaminyl-tyrosine	+	—	+
Polypeptides—			
Leucyl-tri-glycine	—	+	—
18-peptide	—	+	—
Leucyl-glycyl-tyrosine	—	+	+
Leucyl-di-glycyl-tyrosine	—	+	+
Leucyl-tri-glycyl-tyrosine	—	—	+

Professor A. HARDEN, F.R.S. : One well-known example of an adaptive enzyme has been studied in yeast, namely, that by which galactose is fermented by yeast which has acquired the power of fermenting galactose by culture on a medium containing this sugar.

The fact that the rate of fermentation of the fermentable sugars is differently affected by various agents has led to the supposition that at all events some part of the mechanism of fermentation is specific to each sugar. When the dried galactose yeast is washed it loses co-enzyme and becomes inactive and can then be reactivated by co-enzyme from an untrained yeast (Euler and Jansson) so that it is not the co-enzyme, but some factor of the “fermenting complex,” which has been modified or newly produced.

It has long been felt that the sugars undergo some preliminary change by which they become susceptible to the reactions which result in fermentation. Meyerhof has obtained from yeast a substance, hexokinase, which confers on extracts of muscle enzyme the power of rapidly converting hexoses to lactic acid. It seems likely that its function is to convert the hexose into a reactive form and that it may also perform this function in yeast. In presence of phosphate, glucose and mannose are fermented by yeast less rapidly than fructose, but there is evidence that the material actually fermented is the same in all three cases, possibly the enolic form (E. F. Armstrong). Thus

all yield the same hexosediphosphate (Harden and Young) and all yield a mixture of monophosphoric esters of glucose, mannose and fructose (Robison).

It is possibly the rate of conversion of the sugars into this fermentable form which is catalysed by hexokinase (Meyerhof), and the speaker has found that the addition of hexokinase increases the rate of fermentation of all three sugars, and in some cases brings about the fermentation of glucose and mannose as rapidly as that of fructose.

Galactose yields the same hexosediphosphate as glucose (Nilsson) and also yields a monophosphate (Nilsson). The nature of this is not yet known, but the possibility exists that galactose is also converted primarily into the same fermentable material as the other sugars, and that, as suggested by Meyerhof, it is an appropriate hexokinase which is supplied by the adaptive treatment. This possibility is now being experimentally examined.

Professor D. KEILIN, F.R.S.: The main respiratory system of the cell comprises the following components: (1) dehydrogenases, (2) metabolites, (3) cytochrome, (4) oxidase, and (5) molecular oxygen. Of these components, dehydrogenases are enzymes activating the molecules of metabolites which undergo oxidation in presence of a suitable hydrogen acceptor, such as methylene blue or cytochrome. Cytochrome is composed of three distinct hæmochromogens (*a*, *b*, and *c*) each having at its base a different Fe-porphyrin compound. This pigment is very widely distributed in cells of aerobic organisms from bacteria and yeast to higher animals. The oxidation and reduction of this pigment (which is constantly taking place) can be easily seen in living intact cells because, in the reduced state, the absorption spectrum of cytochrome is composed of very distinct bands, while in the oxidised form the absorption bands are diffuse and hardly perceptible. The oxidation of this pigment in living cells, which corresponds to the transition from ferrous to ferric compounds, is affected by the inhibitors such as KCN, H₂S and CO in the same way and to the same degree as the oxidation of *p*-phenylenediamine or of "Nadi" reagent by the intracellular oxidase of the same cells. Moreover, the oxidation of reduced cytochrome is affected by these inhibitors in the same way as the oxygen uptake by the cell containing this pigment. The reduction of cytochrome (*a* and *c*), on the other hand, is inhibited by narcotics such as urethane, which affect the activity of dehydrogenases. Cytochrome in the living cells is oxidised by the oxidase and reduced by the metabolites activated by dehydrogenases.

Of the three components of cytochrome two—*b* and especially *a*—are very

unstable, they are as fragile as enzymes. Component *a*, for instance, in cells warmed above 55° C. decomposes readily, liberating its hæmatin compound which when reduced and combined with bases gives hæmochromogen compounds spectroscopically similar to those which can be obtained from chlorocruorine. On the other hand, component *c* of cytochrome is very resistant, heat stable, and can be easily extracted from yeast cells. The extracted cytochrome *c*, although not oxidisable by molecular oxygen, rapidly undergoes oxidation when brought in contact with oxidase of heart muscle preparation. The oxidase-cytochrome portion of the respiratory system can be easily reconstructed from the oxidase preparation of heart muscle and cytochrome *c* extracted from bakers' yeast; the activity of this reconstructed system can be tested with cysteine. It was found that neither oxidase by itself nor cytochrome alone can appreciably oxidise cysteine. When, however, cytochrome and oxidase are brought together they form a powerful catalytic system rapidly oxidising cysteine to cystine. The activity of this reconstructed oxidase-cytochrome system towards cysteine is destroyed by warming above 70° C., and is inhibited by KCN, H₂S, CO in darkness and CO in light in the same way, and to the same degree, as the respiratory activity of living intact cells. The reconstructed oxidase-cytochrome system behaves therefore like a true respiratory system of the cell. This shows also that the oxidase in heart muscle preparation, that is in remains of dead cells or their extracts, has the same properties and behaves in the same way as in living intact cells.

It is important to note that there are very few aerobic oxidases known in organisms or their extracts. Applying to these enzymes the usual biochemical nomenclature one can enumerate only : (1) indophenol oxidase, (2) polyphenol oxidase, (3) tyrosinase, (4) xanthine oxidase, (5) tyramine oxidase (system ?), (6) glucose oxidase, and (7) urico oxidase. Of these few oxidases only the first three are inhibited with KCN, H₂S and CO ; and only one, the indophenol oxidase which is as widely distributed as cytochrome, is capable of oxidising the reduced cytochrome, and when poisoned by CO can be reactivated with light.

The indophenol oxidase is, therefore, the only aerobic oxidase forming, with cytochrome, an intracellular catalytic system which can be recognised as Warburg's oxidation system of the cell.

Professor J. B. S. HALDANE, F.R.S. : The reaction chain theory of enzyme action propounded by Professor Willstätter presents certain difficulties. If

the chains end by the collision of free radicals we should expect the reaction velocity to vary as the square root of the enzyme concentration instead of being proportional to it. If they include free radicals such as OH and meriquinoids the specificity of enzymes as regards the activation of reducing substrates is hard to explain. Finally, if oxidations in the cell occur mainly in solution and not at surfaces, we should expect their energy to appear as heat, instead of being largely used, as it is, in linked reactions such as the resynthesis of lactic acid to glycogen. However the formulation of this theory, whether or not it is ultimately accepted, cannot but stimulate research, and lead to an examination of basic assumptions.

Among the most important recent advances in enzyme kinetics are the recognition that when reaction velocity is plotted against the logarithm of either hydrogen-ion concentration or substrate concentration we may obtain, on the one hand, a curve rising to a plateau, or on the other a roughly symmetrical curve with a sharp maximum. The plateau type of p_H velocity relation is characteristic of dehydrogenases, as is to be expected on Woolf's theory. The type with a sharp maximum when the substrate concentration is varied appears in the case of a number of enzymes of high affinity, including esterases and dehydrogenases. The low velocities in high substrate concentrations can be explained as due to the formation of an inactive compound between the enzyme and a second molecule of substrate.

An enzyme catalysing the reactions $A + B \rightleftharpoons C + D$ may be specific as regards one, both, or neither of A and B. Thus in the case of yeast maltase, A must be α -D-glucopyranose, B being any one of a large number of alcohols. In the case of malt maltase, B must also be α -D-glucopyranose. Specificity is confined to groups in the substrate molecule which are rigid, such as sugar rings. It never appears to extend to groups which are flexible owing to the possibility of rotation, such as saturated chains. Thus we do not find absolute specificity among the enzymes hydrolysing peptides or esters.

It seems to follow that the part of the enzyme surface responsible for specificity must also be rigid, and therefore probably consists of condensed rings, of which one or more atoms are responsible for the activity. In three enzymes (*Atmungsferment*, catalase, and peroxidase) the catalytic activity is known with high probability to be associated with an iron-porphyrin group, a large, rigid, and approximately flat structure. These three enzymes all catalyse oxidations, but their specificities are different. Their spectra, and hence the structure of the iron-porphyrin group, also differ. Just as the specificity of inorganic catalysts depends on their lattice structure, it is suggested that that

of enzymes depends on the detailed dimensions of their rigid portion. Flexible parts of the molecule, such as peptide chains, are of minor importance.

Dr. J. H. QUASTEL: I wish briefly to describe results which have been obtained recently in an investigation of the effects of dyestuffs on enzymes.

Working with dehydrogenases of bacteria and muscle it has been found that, although a number of dyestuffs have highly toxic effects at low concentrations, the acid dyestuffs are entirely inert. Only basic dyes have inhibitory powers and there is evinced a specificity of behaviour showing that the chemical constitution of the dye, besides its electric charge, plays a part in this phenomenon. Since the activity, however, of bacterial dehydrogenases is, as lysis work has shown, greatly dependent on the integrity of surface structure of the cells, it is impossible to work with these enzymes in a relatively pure and soluble condition, and therefore attention has been turned to other enzymes.

Two enzymes of wide biological distribution and which can be obtained in a highly purified condition were chosen. These were fumarase, which converts fumaric acid into *L*-malic acid, and urease.

Both acidic and basic dyestuffs are highly toxic to fumarase, but great specificity is evinced. Among the basic dyes, those of the triphenyl-methane series are the most active, whilst among the acid dyes, those of the congo red series are most effective. Congo red, indeed, at a molar concentration of 1/100,000,000 will destroy 90 per cent. of the activity of a highly purified fumarase preparation. The toxicity of the dye increases with purification of the enzyme, the presence of proteins leading to combination with the dyes and hence to a protection of the enzyme. It is possible to estimate in this way the combination of dyes with native and denatured proteins. Mixtures of acidic and basic dyes, whether one or both of them be highly toxic or not, at equivalent concentrations, are inert owing to mutual combination or adsorption between the dyes. The toxicity of the dyes might be attributed to combination with charged groups composing the enzyme proper, or perhaps to combination with a colloidal carrier whose structural integrity may be essential to the activity of the enzyme. The substrate molecule, fumaric acid, however, completely protects the enzyme from the toxic effects of low concentrations of the dyes, and this must mean that the dyes combine with groups belonging to the enzyme or to the active centre which constitutes the enzyme. Hence for such combination with both acid and basic dyes to take place the enzyme must contain oppositely charged groups, and it is suggested that fumaric acid,

when combined with or adsorbed by the enzyme, is so arranged that it bridges both oppositely charged groups, in this way affording protection from the dyes. The unsaturated bond of fumaric acid is exposed to the electric field existing between the charged groups, and polarisation followed by activation of the molecule takes place. That there is specificity of attachment of substrate molecules to the enzyme is shown by the fact that only molecules of the same type as fumaric acid, *e.g.*, succinic, malic, or aspartic acid, will afford appreciable protection of the enzyme from the toxic dyes. The specificity of attack by the dyestuffs and their activity at extremely low concentration would indicate the presence of some structure or organisation of groups, which is common to both the enzyme and the dyestuff—neutralisation of electrical charge cannot be the only explanation of this specificity of attack. Experiments with trypanocidally active drugs showed that the *s*-carbamides of the naphthylamine disulphonic acids which are highly substantive to cotton (and, it is to be added, congo red is substantive to cotton) are also highly toxic to fumarase, their effects being specific, so far as is yet known, to this enzyme. The dyestuffs and the drugs appear to combine irreversibly with the enzyme centre, whereas fumaric acid, succinic acid, malic acid and aspartic acid combine reversibly. Presumably only fumaric and *l*-malic acids are so constituted as to undergo activation and subsequent chemical change.

Turning to urease, it was found, using a soya bean extract, that acidic dyes were all quite inert, although the basic dyes of the triphenyl methane series were highly toxic. This was to some extent unexpected, for it was considered that since the enzyme shows an optimum activity at the neutral point, it might be amphoteric in nature and would possess, besides acid groups, basic groups with which acid dyes would combine and render the enzyme inert. This does not, however, occur. Brilliant green is toxic at a concentration of 1/200,000, a concentration at which it is inert with fumarase. Toxicity of the triphenyl-methane series of dyes to urease *decreases* with increased purification of the enzyme, and a highly purified preparation (made from Jack Bean by Sumner's method) showed a 100–200 fold decrease in susceptibility to brilliant green. Soya bean extract contains a substance which enormously enhances the toxicity of the triphenyl-methane series of dyes to the purified enzyme. This substance was found to be contained in the soya bean oil and further work showed that the sensitising effect was to be attributed to the glycerides of highly unsaturated acids such as those found in linseed oil. Moreover, this sensitising effect applies apparently only to the triphenyl-methane series of dyes: it does not apply to such dyes as methylene blue or neutral red, which

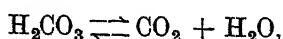
have relatively feeble toxicities. The glycerides in question seem, in fact, to behave as highly specific mordants between the enzyme and dyestuffs. Protection of the enzyme against the dyes is afforded by urea itself and by α -amino acids and basic amines. Amides and hippuric acid do not protect, neither do fatty nor hydroxy nor dicarboxylic acids. The most active group of the urease centre seems to be an acidic or negatively charged one with which α -amino acids and basic amines combine reversibly and basic dyes irreversibly; presumably urea is combined at this group.

Another class of substances combining specifically with urease consists of *o*-dihydroxyphenols and includes catechol, adrenaline and hæmatoxylin. Catechol is toxic to the purified enzyme at a molar concentration of 2 parts in 100 millions. Toxicity increases with purification of the enzyme and is independent of glycerides. The presence of sulphhydryl groups (*e.g.*, thio-glycollic acid, cysteine and glutathione) diminishes or entirely eliminates the toxicity of catechol or adrenaline; this is possibly due to combination with the diphenols. I would suggest that the activating effect of SH groups found in other enzyme systems may be due to neutralisation of natural inhibitors.

The results, as a whole, support the suggestion that the enzymes contain charged groups which will result in the production of local electric fields which may bring about the activation of molecules which are adsorbed there. A certain specific type of substance is adsorbed at the enzyme, but only few of the molecules of this type are so constituted as to undergo activation and subsequent chemical change.

The highly specific toxic effects of dyestuffs and related substances on the purified enzymes, together with a knowledge of the specific classes of substances which protect the enzymes, help to throw light on their constitution and indicate the utility of this method of investigation.

Drs. N. U. MELDRUM and F. J. W. ROUGHTON: When an aqueous solution of bicarbonate is rapidly acidified and the resulting solution violently shaken the rate of escape of carbon dioxide is limited by the velocity of dehydration of carbonic acid,



which goes slowly in contrast to the ionic reactions preceding the formation of H_2CO_3 .

We have recently isolated from blood a new enzyme which catalyses the dehydration of carbonic acid and the hydration of carbon dioxide, and which

thus accelerates the escape of gaseous CO_2 from acidified bicarbonate solutions. This enzyme has been named "carbonic anhydrase."

The first stage in isolation resembles Tsuchihashi's method of preparing catalase: the hæmoglobin being precipitated by shaking with chloroform and alcohol. The filtrate was purified by treatment with alumina cream (alumina C — γ) or calcium phosphate, which do not under suitable conditions adsorb the enzyme. The final product had neither catalase nor peroxidase activity, but still accelerated CO_2 formation even in dilutions of 1 part (dry weight) per 10 millions of solution.

No hæmatin could be found in this colourless preparation, even though the enzyme activity was inhibited by CO (under conditions where inactivation on the surface of gas bubbles was eliminated) and in two out of three cases the CO inhibition was reversibly removed by light. Further investigations along these lines may therefore prove of interest with regard to the meaning of inhibitions produced by CO. Other properties of the enzyme are described in the 'Proceedings of the Journal of Physiology' for March 12 and May 14 (1932).

On the Nature and Permeability of Chitin. I.—The Chitin Lining the Foregut of Decapod Crustacea and the Function of the Tegumental Glands.

By C. M. YONGE, D.Sc., Ph.D., Physiologist at the Plymouth Laboratory.

(Communicated by E. J. Allen, F.R.S.—Received May 21, 1932.)

[PLATE 14.]

I. *Introduction.*

Chitin is widely distributed throughout the animal kingdom and also occurs in the Fungi. Wester (1910) has shown that it is present in all Arthropods, invariably in the exoskeleton and lining the respiratory system and, except in some Arachnids, in the greater part of the gut. In the Mollusca it occurs in the jaws and radulæ, in the shell and sometimes in the gut of Cephalopods and sometimes in the shell and lining of the siphons in Lamellibranchs. The setæ of Annelids are of chitin and occasionally, as in *Lumbricus* and *Aphrodite*, the gut is lined with it. Chitin is also present in the shell, peduncle and spines of some Brachiopods, *e.g.*, *Lingula*, in some Polyzoa, in the Hydroids and very occasionally, as in the gemmules of *Spongilla*, in the Porifera. According to Wester, it never occurs in Protozoa, Echinoderma, worms other than Annelids or in vertebrates. Wherever it occurs, chitin gives the same chemical reactions (Zander, 1897; Wester, 1910) and has the same physical properties, *e.g.*, specific gravity and refractive index (Sollas, 1907; Becking and Chamberlin, 1925) and specific rotation which is always lævorotatory (Irvine, 1909).

It is surprising that so little is known about the properties of this very important substance. In the Arthropods especially, where it covers the entire surface of the body and where respiration and in some cases absorption in the gut—indeed practically all interchange between the interior of the body and the external medium—must take place through it, exact knowledge of the permeability of chitin and of the conditions which control this is clearly essential for a full understanding of the life of the animal. It was the almost complete absence of knowledge on this subject which led to the initiation of this series of researches. Ideal material for the purpose was found in the uncalcified chitin which lines the foregut ("oesophagus" and "stomach") of the Decapod Crustacea, of which relatively large pieces can be obtained

from animals of average size. The lobster, *Homarus vulgaris*, owing to the relatively large size of the foregut, provided the great bulk of the research material, other large Decapods, such as *Palinurus vulgaris*, *Cancer pagurus*, *Maia squinado* and *Carcinus maenas*, being used for comparative purposes.

As so often happens in physiological investigations on invertebrates, the results of the experiments could not be properly interpreted until a full histological examination of the research material had been made. The present paper is concerned with an account of this, and it will be succeeded by papers dealing with permeability. Actually neither the histological nor the physiological side of the work could have been carried far without the assistance of the other.

It is a pleasure to acknowledge the interest and help of Dr. E. J. Allen, F.R.S., and of my colleagues.

II. Structure of the Chitin.

The first accurate account of the structure of the integument in the "Podopthalmous Crustaceans" appears to have been given by Williamson (1860). He describes it as consisting of (a) a superficial, almost structureless pellicle, (b) an areolated layer, (c) a calcified corium, and (d) an inner layer of uncalcified corium which may become calcified as fresh layers appear beneath it. Vitzou (1882), who made a very detailed study of the chitin of Decapod Crustacea, confirmed and extended the findings of Williamson and also reviewed other early work. He examined a great variety of animals, notably *Homarus*, *Astacus*, *Palinurus*, *Maia*, *Carcinus*, *Platycarcinus* and *Portunus*, and found essentially the same conditions in all. He divided the integument into the epidermis, consisting of the integument and the chitogenous epithelium, and the dermis, formed of connective tissue. The chitin of the carapace he divided, like Williamson, into four layers: (a) the cuticle, (b) the pigment layer, (c) the calcified layer, and (d) the noncalcified layer formed of very small lamellæ. In the foregut and hindgut where the chitin is not calcified, he distinguished between the thin, superficial cuticle and the underlying lamellated chitin. His conclusions have been corroborated by later workers, e.g., Herrick (1896) on *Homarus americanus*, and Pearson (1908) on *Cancer pagurus*, and the names employed by him are used throughout this paper.

The chitin from the labrum, oesophagus and stomach of *Homarus vulgaris* have been studied both in microtome and hand sections, the latter being cut between pith and examined in glycerine. Microtome sections of the hind-

gut, gills, and of the uncalcified chitin between the segments of the abdomen, together with sections of various regions in other Decapods, notably *Palimurus* and *Cancer*, have also been prepared for comparative purposes.

In all sections the distinction between the cuticle and the underlying chitin is well shown, figs. 1 and 2, and figs. 8–11, Plate 14. The cuticle is hyaline and has a faint yellow colour. It varies greatly in thickness in different regions, being thickest in the labrum and cesophagus, usually 8 to 10 μ in microtome sections, thinner in the stomach, about 4 μ , thinner again, 2 μ , in the hindgut and excessively thin, not more than 0.5 μ over the gills. The underlying

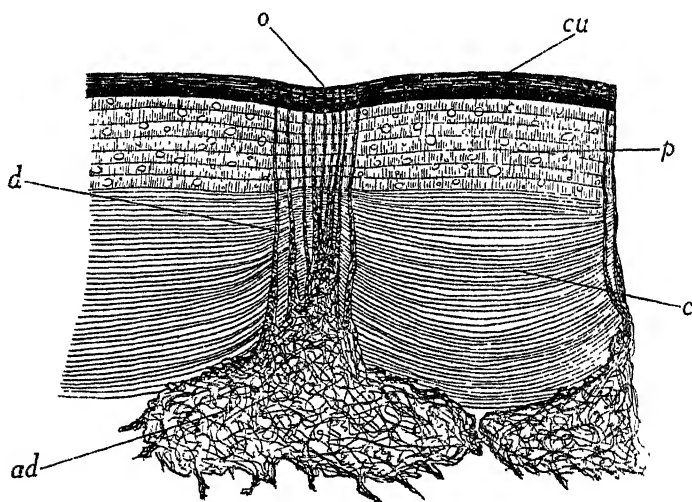


FIG. 1.—Hand section through chitin from anterior portion of cesophagus in *Homarus*. Section treated with $K_4Fe(CN)_6$ at pH 4.2, then with $FeCl_3$ (see Table I). $\times 265$. *ad.*, accumulation of material at base of ducts, stained bluish; *c.*, lower layer of lamellated chitin, not stained; *cu.*, cuticle, stained dark blue as indicated by shading; *d.*, ducts through chitin, stained bluish; *o.*, openings of ducts; *p.*, upper, pigmented layer of chitin, not stained.

chitin is invariably much thicker, ranging from a maximum of some 250 μ in the labrum to a minimum of 10 μ in the hindgut and 4 μ over the gills. It is composed of well-defined lamellæ. Except in the labrum and the anterior region of the cesophagus, where the pigment layer characteristic of the calcified integument is present, it has the same character throughout and is quite colourless. Except in the cesophagus the pigment layer, figs. 1 and 2 and fig. 9, Plate 14, has a slight brown colour in hand sections and is always characterised by the presence of vertical striations and, frequently though not

invariably, rounded inclusions which are responsible for Williamson's term "areolated layer."

Particularly in the labrum and in the first half of the oesophagus, the chitin and cuticle are perforated by series of fine ducts (*d*, figs. 1 and 2 and figs. 8, 10, and 11, Plate 14). These are connected with the tegumental glands which are particularly abundant in these regions.

The morphology and function of these glands are dealt with later in this paper.

The ducts occur in groups of up to 100, which can be demonstrated when pieces of chitin, after appropriate staining, are examined under low power. In

hand sections particularly the ducts are revealed as frequently very irregular in shape with occasional large, rounded swellings, such as are shown in fig. 2 (*sd.*).

The chitin used for hand sections was freed from the underlying tissues by maceration overnight in fresh water.

As a result of this treatment, the continuation and contents of the ducts through the epithelium and connective tissue was also freed from the tissues and, as shown in fig. 1 (*ad.*), forms a tangled mass on the under side of the chitin.

The chitin of the labrum, fig. 9, Plate 14, possesses a few long spines but, apart from clusters of very short projections arising between the openings of

the ducts, the first half of the oesophagus is smooth. At the opening into the stomach and in the stomach itself the chitin is drawn out into series of long, stout spines which are concerned with the passage of food through the stomach and the filtering of the soluble products of digestion from the indigestible residue, the former passing into the hepatopancreas where it is absorbed and the latter being rejected by way of the midgut, hindgut and anus (for details on the physiology of the stomach in Decapod Crustacea, see Jordan (1904, *a*), Williams (1907) and Yonge (1924)). The function of these spines is mechanical

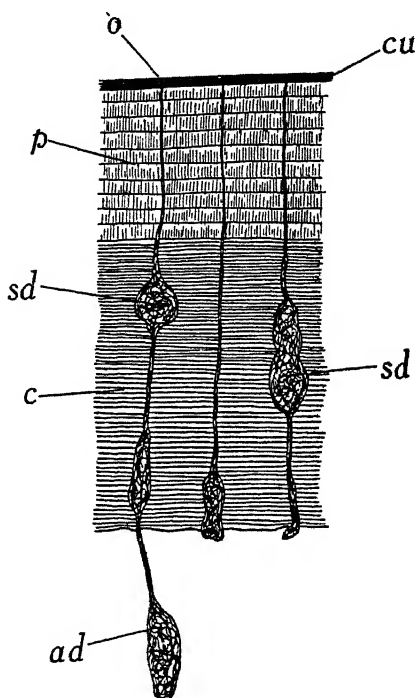


FIG. 2.—Hand section through chitin from labrum of *Homarus*, treated as in fig. 1. $\times 265$. *sd.*, swellings in course of ducts through chitin, stained bluish. Other lettering as before.

only and, unlike the sensory spines which are dotted over the surface of the body and which have been described by Vitzou and Herrick, they have no core of living matter. Vitzou thought that these spines were composed of the same material as the cuticle, but this, apparently obvious, conclusion has not been confirmed by experimental examination, as will be shown later.

The appearance of the spines which protect the ventral groove in the stomach is shown in fig. 3. They arise from pits in the surface of the chitin and, as is

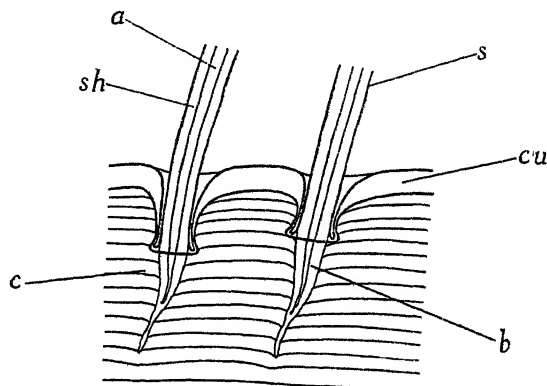


FIG. 3.—Hand section through chitin and spines from region overhanging the ventral groove in the stomach of *Homarus*. $\times 530$. *a.*, axis of spine; *b.*, base of spine which penetrates into the chitin; *s.*, spine; *sh.*, sheath of spine. Other lettering as before.

clearly shown in hand sections but *not* in microtome sections of fixed material, they are continued for some distance downward through the chitin. Each spine consists apparently of two layers, a central axis (*a.*) and a surrounding sheath (*sh.*); sections, fig. 9, Plate 14, confirm this impression. The central axis sometimes has a somewhat granular appearance. Actually, in addition to the axis and surrounding sheath, the cuticle is continued over the surface as a very thin sheet which is invisible in sections, and the manner in which this has been demonstrated will be described in the following section.

III. *Properties of the Chitin and Cuticle.*

Staining Reactions.—Vitzou noted that the staining reactions of the chitin and the cuticle with picrocarmine were different. In the course of this research sections were treated with a variety of stains and in *all* cases the two portions stained differently. Generally speaking, the cuticle stains very readily and the chitin with difficulty. Thus, cuticle stains darkly with iron hæmatoxylin or Delafield's hæmatoxylin, while the chitin stains very slightly if at all; with

erythrosin the cuticle stains dark red and the chitin a faint pink. When a combination of stains is used, very striking differences in the staining of the two portions may be obtained. Thus with safranin and light green the cuticle stains bright red and the chitin green, with Mallory's connective tissue stain the cuticle is coloured a vivid red with acid fuchsin and the chitin deep blue with aniline blue. Both here and with hæmatoxylin the cuticle is stained by the nuclear, *i.e.* basic, dye, while the chitin is coloured by the plasma stains. The ducts through the chitin invariably have the same staining reactions as the cuticle. The spines stain in a very arbitrary manner, and no satisfactory evidence as to their nature can be obtained in this manner. The various staining reactions given by the chitin, cuticle and ducts are summarised later in Table II.

Iso-electric Point.—The difference in the staining reactions is explained when the iso-electric points of the cuticle and the chitin are determined. This was done by the method devised by Loeb (1922), which consists of placing the material in solutions of different hydrogen-ion concentration to which basic or acidic ions have been added. Later, after washing, the range of p_H over which the ions combine with the amphoteric substance is demonstrated by appropriate reactions. Previously this method has been applied to chitin only by Pantin and Rogers (1925), who found that the radula of *Buccinum undatum* consists of a newly formed and an older portion, the former combining with anions and cations irrespective of the p_H while the latter is an amphoteric substance with an iso-electric point at about p_H 2.6. (Incidentally Sollas (1907) found a difference in the action of concentrated HCl and KOH on these two portions of the radula.)

Small pieces of chitin freed from tissue by maceration in fresh water were soaked for 2 hours in 20 c.c. of buffer solution over a wide range of p_H , five drops of 0.1 N $K_4Fe(CN)_6$ being added to each member of one series and five drops of 0.1 N $CuSO_4$ to a second series. After thorough washing the chitin from the first series was treated with a weak solution of $FeCl_3$ which reacted with the anion $Fe(CH)_6'''$ which had combined with the chitin on the acid side of its iso-electric point, giving the typical Prussian blue colour. The other series was treated with $K_4Fe(CN)_6$ which reacted with the cations which had combined with the chitin on the basic side of the iso-electric point to form reddish brown copper ferrocyanide. Hand sections were then made of the chitin and these were examined in glycerine under low power with the results given in Table I.

These experiments show that the iso-electric points of the chitin and the cuticle are quite distinct, the former being about p_H 3.5 and the latter about

Table I.—Iso-electric Points of Chitin and Cuticle from Uncalcified Chitin of the Stomach of *Homarus*.

p_H .	$Fe(CN)_6^{4-}$.		Cu^{++} .	
	Chitin.	Cuticle.	Chitin.	Cuticle.
1.2	Blue	Dark blue	Colourless	Colourless
2.4	Pale blue	"	"	"
3.2	Very pale blue	"	"	"
3.4	"	"	Trace red	"
3.6	Colourless	"	Very pale red	"
4.0	"	"	Pale red	"
4.4	"	Blue	Red	"
4.8	"	Pale blue	"	"
5.0	"	Faint blue	"	Trace red
5.2	"	Very faint blue	"	Pale red
5.4	"	Colourless	"	Red
5.8	"	"	"	"
6.2	"	"	"	"
7.2	"	"	"	"
8.0	"	"	"	"
9.0	"	"	"	"

p_H 5.1, the former being nearer to that found by Pantin and Rogers for the old chitin of the radula of *Buccinum*. No easier method for demonstrating the difference between the cuticle and the chitin was found than making sections of material treated with $K_4Fe(CN)_6$ and $FeCl_3$ at p_H 4.2 when, as shown in figs. 1 and 2, the cuticle appeared dark blue and the chitin colourless. The contents of the ducts also coloured a pale blue, but the spines remained colourless. An attempt was made to discover whether the new chitin had no iso-electric point, *i.e.*, was not amphoteric, by treating in the same manner new chitin from an animal in the early stages of ecdysis, but invariably the same iso-electric points for the chitin and the cuticle were found. Similar results were obtained with the uncalcified chitin from between the segments of the abdomen.

Action of Acids and Alkalies.—Chitin is an amino-polysaccharide, the exact chemical composition of which is unknown, although when hydrolysed with strong acids it yields glucosamine and acetic acid, and also some sulphuric acid. It is supposed that the amino groups of the glucosamine are largely acetylated. It dissolves in concentrated mineral acids but not in alkalies, although after boiling with the latter the chitin is converted into chitosan. Chitosans give a vivid violet colour when treated with a weak solution (0.2 per cent.) of iodine with 1 per cent. H_2SO_4 . This reaction was employed by Wester in his studies on the distribution of chitin, his procedure being to heat sections of chitin with

60 per cent. KOH in an oil bath at 160° C. for 10 to 20 minutes. The sections were then transferred to a watch glass containing alcohol which was gradually diluted until the sections were in water, when they were treated with iodine and examined under the microscope.* Wigglesworth (1929) has employed the same method to demonstrate the presence of chitin in the gut of *Glossina*.

The same procedure was followed in this research, hand sections of the chitin of *Homarus* being treated with concentrated solutions of NaOH and KOH in a boiling water bath for from 10 to 20 minutes. The violet colour was obtained with iodine and H_2SO_4 , but when the sections were examined it was discovered that *the cuticle had completely dissolved*. This was repeatedly confirmed. The appearance of a section through the spinous region which overhangs the ventral groove in the stomach after this treatment is shown in fig. 4. The cuticle has

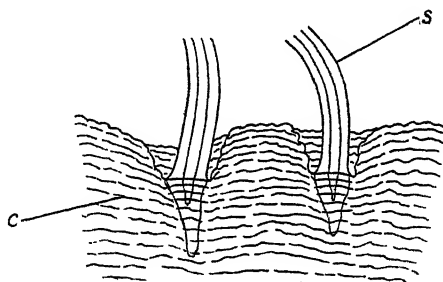


FIG. 4.—Hand section similar to that shown in fig. 3 after 10 minutes in boiling concentrated NaOH which entirely removes the cuticle, the chitin appearing more opaque and the lamellæ becoming wavy and irregular instead of straight. $\times 530$. Lettering as before.

disappeared, the spines remain, apparently intact with the two layers well defined but without their normal rigidity (compare fig. 3), while the chitin is also intact but the lamellæ have become wavy and irregular and the surface is thrown into small folds. Sections of the labrum and cesophagus treated in the same manner revealed that not only the cuticle had dissolved but also the mass of material at the base of the ducts, fig. 1, *ad.*, while the ducts themselves were almost obliterated. Owing to its dissolution it was impossible to apply the iodine test to the cuticle, but the very fact that it does dissolve under such conditions indicates that it is *not* of the same nature as chitin.

* This method was first used by Van Wisselingh (1898) and has recently been further improved by Campbell (1929). I have to thank Dr. V. B. Wigglesworth for drawing my attention to this important paper by Campbell, which also contains a valuable summary of previous work on Insect chitin.

Hand sections of chitin were then treated with concentrated HCl in the cold. As was expected, the actual chitin dissolved almost immediately, but the cuticle was much more resistant to the acid. Fig. 5 shows the appearance of a hand section of the chitin from the oesophagus after it had been immersed in concentrated HCl for $2\frac{1}{2}$ hours. The cuticle, ducts and accumulated material at their bases are all intact, but the actual chitin has been completely dissolved. The action of HCl is thus the precise opposite to that of boiling alkalis. Hand sections from the spinous regions in the stomach were treated

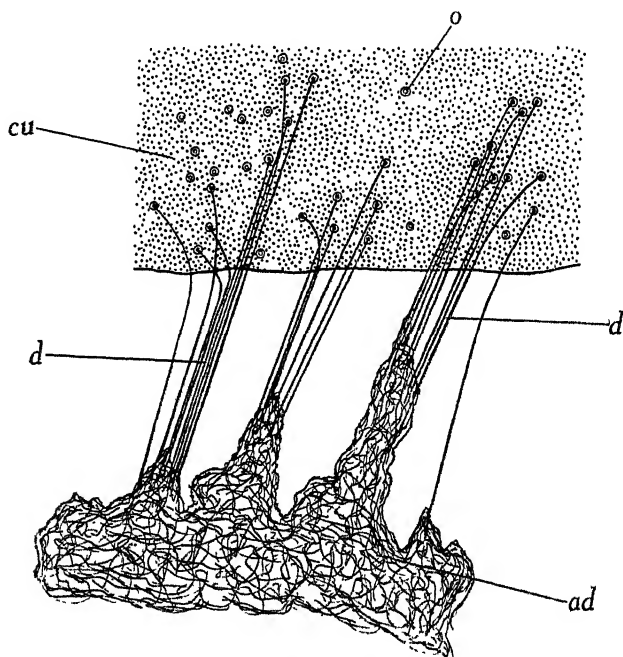


FIG. 5.—Hand section similar to that shown in fig. 1 after $2\frac{1}{2}$ hours in cold concentrated HCl, the chitin being entirely dissolved but the cuticle, contents of ducts and the accumulation of material at their bases, being still intact. $\times 530$. Lettering as before.

in the same manner. After 1 hour the chitin had largely dissolved, while the cuticle in many places had broken away from the underlying chitin, pulling with it the spines with their basal projections into the chitin. Half an hour later there was hardly any chitin left but the cuticle was intact. The spines were intact and rigid, unlike those treated with alkalis, but their basal projections had been dissolved. These, therefore, are of chitin and not formed from the cuticle. After 5 hours conditions were about the same, no trace of chitin remaining. After 7 hours the cuticle had been dissolved to some extent.

while the spines were in process of dissolution. They had lost their rigidity owing, as examination revealed, to the dissolution of the thin protective covering of cuticular material. This is shown in fig. 6, where two spines are illustrated in which the thin cuticle is in process of dissolution, exposing the chitinous material beneath: the later has lost its typical appearance, become irregular and is being dissolved (*dc.*). The spines swell greatly before final dissolution, and their nature is proved conclusively by the experiments with alkali and acid. In alkali the thin covering of cuticle is lost and the spines, while retaining their thickness and apparent structure, lose their rigidity.

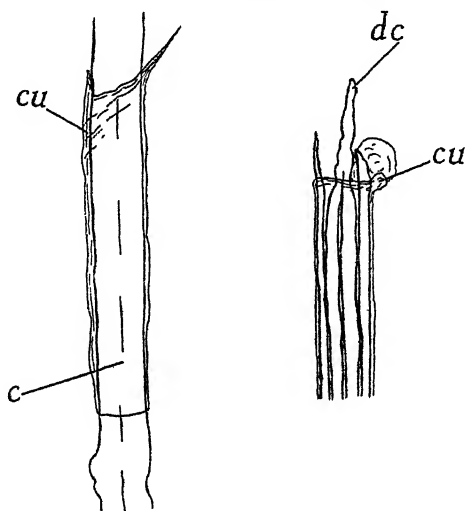


FIG. 6.—Spines from the stomach of *Homarus* after 7 hours in cold concentrated HCl. $\times 530$. *dc.*, dissolving chitin. Other lettering as before.

In acid the thin cuticle protects the chitin, although the basal projections are dissolved. Finally, when the cuticle itself disappears the spines rapidly dissolve in acid. This was confirmed by treating sections first with boiling concentrated NaOH for 20 minutes and then with concentrated HCl in the cold. The cuticle was removed by the first treatment and as a result the spines were very quickly dissolved by the acid. Concentrated HNO₃ and H₂SO₄ in the cold, and also Schulze's reagent (equal parts concentrated HNO₃ and saturated solution of KClO₃ in water) have the same differential action on the chitin and cuticle.

Action of Chitinase.—Karrer and Hofmann (1929) have shown that there is an enzyme in the gut of snails which, at an optimum p_H of 5.2, converts chitin into N-acetylglucosamine with traces of glucosamine. Hand sections of

chitin were treated with fluid collected from the stomachs of the common *Helix aspersa*, toluol being added as an antiseptic, and the presence of this chitinase was confirmed. But the chitin *alone* was attacked, both the cuticle and contents of the ducts being unaffected. This provides yet further evidence that the cuticle has *not* the same chemical composition as chitin.

Presence of Lipoid.—Experiments on the permeability of the chitin from the stomach of *Homarus*, which will be described in a later paper, gave results which could be explained only on the assumption that some portion of the integument contained lipid. To obtain histological confirmation of this and to determine the distribution of the lipid, sections were fixed and stained according to Ciaccio's lipid method. Surprisingly definite results were obtained. In no case was any red colour seen in the chitin, but *the cuticle invariably gave a positive reaction for lipid*, a faint and diffuse but decided pink. The appearance of such sections is shown in fig. 9, Plate 14, where the cuticle of both the old and the new chitin was stained a distinct pink. The ducts through the chitin also stain pink. The great affinity of the cuticle for stains might have been the explanation for the pink colour. Experiments, however, proved that this was not the case. Before Ciaccio fixation pieces of chitin were treated for 4 days with (a) absolute alcohol, (b) ether (after dehydration in acetone), (c) chloroform (after dehydration), (d) methyl acetate, (e) acetone and, for 2 days, with (f) N HCl, and (g) N NaOH. Sections were subsequently prepared and the cuticle stained a decided pink *only* in (d) and (e), *i.e.*, in the two organic solvents which are *not* lipid solvents, according to Maclean (1918) in his account of the properties of lecithin. The three lipid solvents, (a), (b) and (c), and the strong acid and alkali which would hydrolyse lipids, each remove practically all trace of staining from the cuticle. Similar results were obtained with Sudan III which stains the cuticle on untreated hand sections a bright pink. It can, therefore, be stated with confidence that the cuticle contains a lipid, possibly closely allied to lecithin, which is presumably adsorbed upon it. The supreme importance of this lipid in regulating the passage of substances through the cuticle, and so through the whole of the chitin, will be shown in later papers.

Water Content.—The water content of chitin is very high. Rough estimations were made by drying large pieces of chitin, macerated in sea water, between filter papers and then weighing them. Different samples varied, the chitin being thicker in some regions than others, but an average of four estimations gave an increase in weight of 43·7 per cent. when the chitin was transferred to fresh water from sea water and a decrease to 22·9 per cent. of the original

weight after the chitin had been dehydrated in absolute alcohol and then dried in a desiccator. Hand sections were examined first in fresh water and then in absolute alcohol, and the diminution in width of both the cuticle and the chitin appeared about the same, to between 70 and 80 per cent. of the original width. It would seem, therefore, that the two have approximately the same capacity for imbibing water.

IV. *Distinct Nature of the Cuticle.*

With the exception of the water content, all the foregoing experiments on the properties of chitin indicate that the cuticle has a distinct nature from the underlying chitin. Moreover, when sections are examined under the highest powers, figs. 8, 9 and 11, Plate 14, it can be seen that the chitin immediately beneath the cuticle is not perfectly smooth but is somewhat irregularly scalloped, whereas, except when it is old and has become worn, the free surface of the cuticle is invariably perfectly smooth. Again, when the cuticle is cut in sections it breaks away from the chitin beneath and usually curls back, as shown in fig. 7, indicating that it is normally in a state of tension. The chitin never behaves in this manner, but merely frays out.

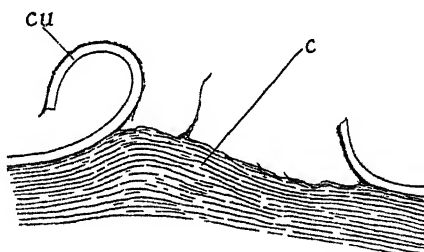


FIG. 7.—Microtome section of the chitin from the cesophagus of *Homarus* showing the effect of a tear, the cuticle breaking away from the underlying chitin and curling back. $\times 530$. Lettering as before.

It will be of advantage now to summarise the differences between the cuticle and the chitin before proceeding to an examination of their origin. Eight distinctions between the two have been described :—

- (a) The cuticle is hyaline and structureless, the chitin is composed of parallel lamellæ.
- (b) The cuticle stains more readily than the chitin and has a special affinity for basic stains.
- (c) The cuticle has an iso-electric point at p_H 5.1, the chitin at p_H 3.5.

- (d) The cuticle dissolves in boiling concentrated alkalies, the chitin does not.
- (e) The cuticle is much more resistant to the action of cold concentrated mineral acids and Schulze's reagent than is the chitin.
- (f) The cuticle is not attacked by the chitinase from *Helix*.
- (g) The cuticle possesses adsorbed lipoid, the chitin does not.
- (h) The cuticle is normally in a state of tension, the chitin is not.

This extensive body of evidence indicates that the cuticle is *not* chitin and that it has a distinct origin from the chitin over which it is spread out as a thin sheet. The question then arises, is there any agency which could be responsible for this? Immediately there comes to mind the fact that the contents of the ducts through the chitin possess all the properties of the cuticle with the exception of the last, for which there is no means of testing. They have the same yellowish colour, the same staining reactions, iso-electric point, same reactions to alkalies, acids and chitinase, and they contain lipoid. These ducts are the outlets of the secretion from the tegumental glands which will now be described.

V. *The Tegumental Glands.*

The tegumental glands consist of small, rosette shaped organs which are distributed everywhere beneath the surface of the integument, in the foregut and hindgut and in the gill chamber as well as over the entire surface of the body, in all Decapod Crustacea. They have been described by many workers. Braun (1875, 1877) noted the presence of these glands under the integument and in the foregut of a number of Decapods. Vitzou (1882) found them in the oesophagus and hindgut of all the Decapods he studied and states that they are characteristic of all the higher Crustacea. Frenzel (1885) and Costes (1890) described these glands in the chitinised regions of the gut in various Decapods. Allen (1893, *a*) was the first to report their presence in the gills when working on *Palæmonetes varians*, and later (1893, *b*) he made the important observation that the day-old larva of this species possesses a single pair of very large glands the ducts of which open at the base of the first maxillæ, while there are numerous smaller "salivary" glands in the labrum, paragnaths and maxillæ. Cuénot (1895) confirmed the presence of the glands in the gills and also in the foregut and hindgut of a number of Decapods. Herrick (1896) states that they are distributed everywhere under the integument of *Homarus americanus* and that they are especially numerous in the foregut, hindgut and in the pleopods of the female. Wallengren (1901), by staining the chitin of the foregut and

hindgut so as to demonstrate the presence of the ducts, described the distribution of these glands in these regions. Apáthy and Farkas (1908) were the first to make a detailed histological study of the glands, using those from the hindgut of *Astacus*, and Farkas (1914, 1927) has continued this, working on the glands from the oesophagus of *Astacus*. In his last paper he has given a very detailed and accurate account of the histology of these glands. Tegumental glands have also been described in *Cancer* by Pearson (1908), in the *Paguridae* by Issel (1910), in *Eupagurus* by Jackson (1913) and in the hindgut of *Astacus* by Janisch (1923) and in the foregut and hindgut of *Nephrops* by Yonge (1924).

Description.—The tegumental glands have been described in such accurate detail by Farkas (1914, 1927) that it is only necessary here to draw attention to their general characteristics. In *Homarus* each gland, figs. 12–14, Plate 14, consists of a spherical cluster of secreting cells (*g.c.*) with basally situated nuclei (*n.g.*) which degenerate in the old glands (*d.n.g.*). In the mature gland, fig. 13, fine capillary ducts (*ca.*) ramify through the substance of the cells uniting, at the narrow apex of the cells, with a series of collecting ducts (*c.d.*) which pass through a fibrillar substance that occupies the centre of the structure and open into the large main duct (*m.d.*). These ducts are formed by large amoeboid cells (*d.ce.*) which have characteristically large nuclei (*n.d.*) each with a conspicuous nucleolus. The ducts, which have comparatively thick walls, are formed intracellularly within the duct cells, the nucleus of one of these being invariably present within the actual gland, as shown in figs. 12–14. Herrick mistook this for the nucleus of a bipolar nerve cell, an error which Farkas has pointed out. The remainder of the ducts is formed in the same manner, the number of cells depending on the distance the gland is situated from the epithelium. The ducts pass through the connective tissue and the epithelium and are continued through the chitin as already shown, figs. 1 and 2.

Each gland is surrounded by a very delicate capsule of connective tissue which is not shown in the figures. According to Apáthy and Farkas the central fibrillar substance round which the gland cells are grouped and the collecting ducts which pass through this, are formed by the innermost duct cell, but Janisch believes that they are produced by the actual gland cells. The former view appears to be the correct one; the staining reactions of this region and of the walls of the ducts are the same and both can be freed from the tissues by maceration. Herrick has described and illustrated this and his results have been confirmed, using Bélla Haller's maceration fluid.

The tegumental glands in the alimentary canal of *Astacus*, as described by

both Farkas and Janisch, consist of elaborate ovoid or spheroid structures with ducts running through them subdividing and penetrating into their various ramifications, the whole structure attaining a maximum diameter of about 500 μ . The tegumental glands in *Homarus*, on the contrary, are always spherical, simple in structure and never exceed a diameter of about 80 μ . On the surface of the body in *Homarus americanus* Herrick describes and figures them as occurring singly, but in the œsophagus of both species they occur in groups of 20 or many more, closely packed together, the entire group being compressed at right angles to the epithelium. Each of these groups probably corresponds to one of the more complex glands in *Astacus*. The collections of openings on the cuticle numbering, as already noted, from 20 to 100, apparently each represent the outlets of a single group of glands. In *Astacus*, as shown by Wallengren (1901), these openings occur only in groups of three or four together, which reflects the greater complexity of the glands to which they give outlet.

Wallengren made a comprehensive study of the distribution of the openings of the glands in the alimentary canal in a variety of Decapods, including *Homarus*. He found that if the chitin was treated for 10 minutes in the dark with silver nitrate, then washed and exposed to the light, the cuticle (owing, though he did not then realise it, to its more alkaline iso-electric point) took on a brown colour, the underlying chitin remaining colourless while the openings of the ducts appeared as distinct, dark spots. He found that in *Homarus* tegumental glands are very numerous in the foregut, especially on either side of the labrum, in the lateral lips and in the wings of the metastoma and, most abundant of all, in the anterior half of the œsophagus. They were very sparsely distributed throughout the posterior half of the œsophagus, completely absent in the stomach but plentiful in the hindgut, especially, as sections show, in the swelling at the junction with the midgut. Wallengren's observations have been confirmed, some evidence of the abundance of the glands in the œsophagus being provided by the fact that, in the region of their greatest concentration, about 250 have been counted in sections 6 μ thick beneath 1 mm. of the epithelium.

Theories as to their Function.—It is surely obvious that glands which are so abundant and so ubiquitous in every Decapod which has been examined, and also in lower Crustacea, must play a very important rôle in the lives of these animals. A number of authors have contented themselves with a description of the glands and put forward no views as to their function, but others have advanced various theories which may be summarised as follows :—

(a) *Salivary Glands*.—Owing to their great abundance in the labrum and oesophagus, it was maintained, first by Braun (1877) and later by Vitzou, who, however, admits that it is very difficult to pronounce categorically as to the function of the glands, and by Frenzel, Wallengren, and Pearson, that the glands in this region produce a salivary secretion, although they admitted that the glands in other regions must have a different function. Actually no salivary secretion is necessary in the Decapods, food passing straight through the oesophagus into the stomach, where it is acted upon by the secretion from the hepatopancreas which contains enzymes which act on proteins, fats and carbohydrates.

(b) *Slime Glands*.—The majority of workers have fallen back on the somewhat non-committal theory that the tegumental glands produce some form of slimy secretion, this view being held by Wallengren (for the glands from the hind-gut), Jackson, Janische and Farkas (1927). The statements of the last-named will be discussed at more length later.

(c) *Sense Organs*.—Herrick, basing his view largely on those of Patten (1894), who had studied similar glands in *Limulus*, thought that the tegumental glands on the labrum and others regions about the mouth had a gustatory function, though he admits that it would "be absurd to suppose that the apparently similar organs in many other parts of the body, as in the carapace, possessed a similar function." He regarded the tegumental glands as very primitive organs in the Arthropods and which had been "modified to perform different functions with a minimal change of gross anatomical structure." The conclusions arrived at in the present paper indicate that the tegumental glands produce the same secretion wherever they are situated, but whether this view meets with general acceptance or not, there is certainly no justification for regarding such typical glandular structures as sense organs.

(d) *Excretory Organs*.—Lang (1891), on the evidence that carmine injected into the body might be subsequently found in the tegumental glands, regarded them as excretory organs. It is, however, a common experience to find colouring matter taken up by secreting cells, such as those in the hepatopancreas. This, as shown by Jordan (1904, b), is no indication that these cells possess any excretory functions.

(e) *Cement Glands*.—The great abundance of the tegumental glands in the pleopods of female decapods originally led Braun (1875, 1877) to the conclusion that they formed the cement which binds the eggs to the appendages. This view found support later from Cano (1891) and Herrick, the latter bringing forward the definite evidence that the glands here are full of secretion imme-

diately before oviposition, while many, though not all, are empty after the eggs have been shed. It is not intended in this paper to go into this matter in any detail, but it may be stated that preliminary experiments *do* indicate that this view is correct. Such a conclusion, moreover, agrees entirely with the general results of this enquiry into the function of the tegumental glands.

(f) *Other Theories*.—It is remarkable that the *invariable association between tegumental glands and chitin* has not been commented upon by other workers. Although the relative lengths of the midgut and the hindgut in the Decapods vary very greatly—in *Homarus*, *Nephrops* and *Paguristes* the midgut is very long and the chitinated hindgut extremely short, whereas in *Astacus*, *Cancer* and the great majority of Decapods, the midgut is extremely short, only a few millimetres in length—yet the tegumental glands are *always* confined to the hindgut. It was this fact in particular which led the author (1924) to advance the theory, as an alternative to the view that they produced a sticky secretion, that the tegumental glands in Decapods might have “some function connected either with the secretion or preservation of the chitinous lining.”

The similarity between the properties of the cuticle and the contents of the ducts through the chitin which come from the tegumental glands has already provided evidence that this last view is well founded. For definite proof a study of the process of formation of the new integument before ecdysis and the association with this of the tegumental glands is necessary.

VI. *The Formation of the New Integument.*

Methods of Investigation.—Amongst the Decapods, *Homarus*, in common only with *Astacus*, presents unique advantages for the study of the formation of the integument. This is due to the presence, during the period before the moult, of gastroliths or “crab’s eyes.” These organs have been a subject of interest to naturalists from very early times, a full account of the literature on the subject being given by Herrick. They consist of lens-shaped concretions of calcium carbonate which develop, one on either side of the stomach, between the new and old chitin in animals which are preparing to moult. In a fully-grown lobster they may attain a diameter of over 2 cm. and a thickness of 5 mm., and are made up of great numbers of thin, truncated prisms. At the time of the moult they fall into the cavity of the stomach and in due course are broken up by the gastric mill and passed through the gut. There have been many theories as to their function, all of which are reviewed by Herrick, but the theory of Herrick himself appears to be correct. Before the moult

the calcification in many parts of the shell, notably down the mid-dorsal line of the carapace, is reduced, presumably to assist in the breaking away of the old shell. Herrick believes that the gastroliths represent the lime which has been removed from the shell, and which is accumulated and subsequently ejected. He states that in the *Brachyura*, where there are no gastroliths, there is much less absorption of lime from the shell, while in the lobster the absorption of lime proceeds *pari passu* with the growth of the gastroliths. Certainly the statement (which has been confirmed) that they are cast into the stomach at the moult would appear to dispose of the old theories that the gastroliths are a reserve of lime for the formation of the new shell.

The presence of gastroliths proved of the greatest help in this research. When animals were opened to obtain chitin for experiments on permeability, a careful watch was kept for gastroliths and where these were present portions of the labrum and of the anterior half of the oesophagus were removed and fixed, Bouin's, Flemming's and Ciaccio's fluids being principally used. The condition of the gastroliths was noted. When first discernible they consist of small crystals which form opaque whitish patches on either side of the stomach. Later these coalesce and grow in thickness, though not in area, until they attain full size. It is unknown exactly how long they take to develop. The fixed material was labelled "very small," "medium," "large" or "very large gastroliths," according to the state of these structures. Sections were subsequently prepared 6 μ thick and were stained in a variety of ways as noted later. In the absence of the gastroliths it would have been impossible to have obtained the early stages of formation of chitin without fixing and sectioning a very great quantity of material.

Appearance of New Chitin.—There is no definite evidence of the formation of new chitin until the gastroliths are of medium size, *i.e.*, about half formed. When the gastroliths are forming (and also for some time before this) the old chitin appears no longer to adhere closely to the epithelium, but closer examination shows that portion next to the epithelium has become altered in texture, *d.c.*, figs. 8 and 9, Plate 14. It has lost its regular lamellated structure, become irregular and open in texture and only stains very light blue with Mallory. Nuclei, *n.d.c.*, fig. 8, Plate 14, smaller than those of the epithelial cells are abundant in this region, though, possibly because it has the same staining reactions as the surrounding chitin, no cytoplasm can with certainty be distinguished. When the gastroliths attain medium size, fig. 8, Plate 14, the under surface of the old chitin presents an irregular appearance, the region of altered chitin extending in places deeply into it. Wherever this occurs the apex of the

indentation is invariably occupied by one or more nuclei, as shown in fig. 8, and there seems no doubt that these are responsible for a softening and final dissolution of part of the under surface of the chitin, this being an indispensable aid to the formation of new chitin, which it invariably precedes. So far as can be found this is an original observation for the Crustacea, Vitzou merely reports the presence of a gelatinous layer between the old and the new chitin. In the Insecta, Tower (1906) has shown that, in addition to the fluid which the exuvial glands are supposed to pour out between the two layers of chitin, there is an actual dissolution of the under surface of the old chitin. In this case there are no nuclei, but an enzyme which dissolves chitin is apparently secreted by the epithelium. Buddenbrock (1930) comes to a similar conclusion, even regarding this as the *sole* source of the exuvial fluid. In its final stages in the Decapods the softened chitin loses all structure, fig. 9, Plate 14, becoming, apparently, very fluid, and its staining reactions often resemble those of the cuticle rather than of the chitin.

New chitin, *n.c.*, fig. 8, Plate 14, first appears as a thin layer no more than $2\ \mu$ thick when the gastroliths are of medium size, but there is *no cuticle*. Careful examination of sections reveals, however, the presence here and there of secretion appearing over the surface of the chitin from the ducts of the tegumental glands (*s.d.n.*, fig. 8). When the gastroliths are large, sections show conditions which are illustrated in fig. 9, Plate 14. The old chitin (*o.c.*), which is bounded by a cuticle some $7\ \mu$ thick, is separated from the new chitin by a layer of softened chitin about $40\ \mu$ wide. (The nuclei are not shown because this section was stained only with Ciaccio's lipid stain.) The new chitin consisted of a cuticle (*n.c.*) not more than $3\ \mu$ thick and underlying chitin about twice as wide. Both the old and the new cuticles stained a distinct red with Ciaccio's lipid stain. The area of softened chitin contained many spines (*n.s.*) which had been cut transversely owing to the fact that they are unable to erect themselves fully while the old chitin remains. The section was cut through the spinous region of the labrum. The point of greatest importance here is that, although the new cuticle is now well developed, it is still *less than half as thick as the old cuticle*. In other words it must either be formed from the underlying chitin—a conversion which would involve a complete change in physical and chemical properties and the acquisition of adsorbed lipid—or be laid on by some agency from beneath. The latter is clearly the only feasible alternative.

Later stages show a continual increase in the thickness of both the cuticle and the chitin. In an animal with fully formed gastroliths the cuticle was

about 5 μ thick and the chitin between 40 and 60 μ , fig. 11, Plate 14. At this stage the old chitin invariably comes away during fixation. The same conditions were found in *Palinurus*, the animal, which was about to cast its shell, possessed on the œsophagus, fig. 10, Plate 14, new chitin 12 μ thick covered by a cuticle 5 μ wide, whereas the old chitin (not shown in the figure) was about 70 μ thick with a cuticle 10 μ in width.

There is thus definite evidence that the underlying chitinous layer is the first formed, that the cuticle appears first as a thin layer over it and that the two then increase in thickness simultaneously until the integument is completely formed. Vitzou also noted the extreme thinness of the cuticle in the new chitin and the fact that the underlying chitin was much better developed; this he shows, though not very distinctly, in his figures. He did not comment, however, on this observation and, in view of his ignorance of the very different properties of the cuticle and the chitin—which are here indicated for the first time—he presumably regarded the structureless cuticle as the result of cornification of the lamellated chitin.

It is now necessary to examine the mode of formation of both the chitin and the cuticle, which involves a study of the functions of the chitogenous epithelium and of the tegumental glands.

Function of the Chitogenous Epithelium.—Previous to Vitzou's investigations there were two views as to the formation of chitin. One was to the effect that the entire epithelial cells were converted into chitin and the other that these cells actually secreted chitin. Vitzou disagreed with both theories and advanced a third which appears to be correct. He stated that the upper portions of the epithelial cells become modified, forming chitin which is then separated from the rest of the cell. This process is continued, each layer representing a lamella in the fully formed chitin. In the majority of Decapods the chitin from adjoining cells does not fuse readily and, as a result, the chitin is traversed with vertical as well as horizontal lines. The whole of the chitin, in Vitzou's opinion, thus consists of a series of minute, multi-layered cylinders lying closely packed side by side, and each one representing the activities of a single epithelial cell. In some cases the chitin from adjoining cells fuses much more readily than in the majority, and in these the vertical lines are absent or very difficult to distinguish. *Homarus*, as Vitzou noted, happens to be an extreme example where fusion takes place very readily, and it is impossible to distinguish vertical striations except at the very beginning of the process of chitin formation, fig. 8, *n.c.*, Plate 14. In *Palinurus* the vertical striations are very easily distinguished and these are illustrated in fig. 10, Plate 14 (*v.s.*).

Vitzou also states that during the formation of new chitin the epithelial cells assume gigantic proportions, later diminishing again. Measurements of the oesophageal epithelium in lobsters in various stages of gastrolith formation gave the following average figures :—no gastroliths, 95 μ ; very small gastroliths, 100 μ ; medium gastroliths, 120 μ ; large gastroliths, 170 μ ; very large gastroliths, 110 μ . This agrees entirely with findings recorded in the previous section, chitin formation beginning when the gastroliths attain medium size and being probably at its maximum when they are large, the effect of chitin formation being reflected in the diminution of the width of the epithelium when the gastroliths attain their full size.

The results of the present research are in entire agreement with those of Vitzou so far as the formation of the actual chitin, though not of the cuticle, is concerned. The chitin is formed by the chitogenous epithelium, the terminal portions of the cells apparently forming successive thickenings, each of which represents a single lamella of the chitin, the cells increasing greatly during the process. The glycogen which accumulates in the hepatopancreas is transferred by way of the blood stream to the epidermis immediately before the moult and there utilised by the epithelium for the formation of chitin (Bernard, 1853 ; Kirch, 1886).

Function of the Tegumental Glands.—Both the identity in properties of the cuticle and the contents of the ducts through the chitin, and the fact that the cuticle is not formed before the underlying chitin but first appears after the chitin and then increases with it, point to the tegumental glands as the source of the cuticle. It remains to be seen what additional confirmation of this view can be obtained from a study of the relationship of the activities of these glands to ecdysis, and an examination of their staining reactions.

Farkas (1927) believed that the activities of the glands can be divided into various stages, but that they never return to a former condition, as in the majority of glandular structures, but eventually degenerate. He distinguished between the following four periods in the life of the glands :—

1. Period of construction, in which secretion forms, the capillary canals develop, and the nucleus moves from the centre of the cells to the base.
2. Period of rest, in which there is no extrusion of secretion but the cells are reorganised, the capillary canals are very small, and there is much glycogen in the cells.
3. Period of reconstruction, in which new secretion is formed and capillary canals reappear in the cells.

4. Period of destruction, in which secretion is active, the capillary canals ramifying through the cells. In the final stages the nuclei degenerate, the capillary canals are reduced to stumps, the cytoplasm is very vacuolated, and the entire cells are ultimately destroyed.

In the course of their various activities Farkas states that the glands secrete a mucus-like substance, a substance containing glycogen, a chitin-like substance and "Drusenwasser." No attempt has been made to repeat his elaborate staining methods, his interpretation of the results of which must be largely a matter of personal opinion.

What has been done is to correlate the activities of the glands with ecdysis, and a definite result has been obtained. The gland cells stain blue with Delafield's hæmatoxylin, red with safranin, blue with Mallory; in the young cells, fig. 12, Plate 14, the stain is invariably pale and diffuse, the old, degenerating cells, fig. 14, Plate 14, are very vacuolated and stain very palely. As they develop the contents of the gland cells stain very vividly, fig. 13, Plate 14, *g.c.s.* In the period between the moults it is always possible to find a certain number of glands some of whose cells stain very darkly. Conditions remain much the same at the stage when very small gastroliths are present: there are more general signs of secretion when the gastroliths have attained medium size, when the cuticle first appears, and secretion is everywhere intense when the gastroliths are large, cuticle formation being then at its height. At this stage also many glands have discharged their contents, are empty with vacuolated cytoplasm and nuclei reduced or absent and beginning to degenerate, such as the gland shown in fig. 14, Plate 14. Alongside them are other glands which are just beginning to form, and consist of little else but a concentration of large nuclei; others, such as the one shown in fig. 12, have assumed shape, but are small with relatively large nuclei and a main duct, but the collecting ducts are difficult to distinguish. When the gastroliths are very large only a few glands remain in an active state of secretion, with some of their cells discharged and empty (fig. 13, *g.c.e.*), the others being in various stages of degeneration, while there is an abundance of young glands.

At no other stage except immediately before the actual moult are degenerating and newly formed glands seen, and at no stage are the signs of secretion so widespread. There can be no question but that the glands are intimately connected with ecdysis, at the conclusion of which they all degenerate and are destroyed, new glands forming which develop between the moults, and show the greatest indications of secretion at the time when the cuticle is being most rapidly formed

The histological and histo-chemical evidence as to the nature of the secretion and its relation to the contents of the ducts and to the cuticle and chitin is largely summarised in Table II.

Table II.—Staining Reactions of Secretion in Tegumental Glands and Ducts and of Cuticle and Chitin.

Stain.	Secretion of glands.		Ducts through chitin.	Cuticle.	Chitin.
	In cells.	In ducts.			
Safranin and light green Delafield's hæmatoxylin	Red Blue	Red Blue	Red Blue	Red Blue	Green Almost colourless*
Iron hæmatoxylin	Black if stains	Black	Black	Black	Colourless
Toluidin blue	Blue	Blue	Blue	Blue	"
Erythrosin	Red	Red	Red	Red	Faint red
Mallory	Blue	"	"	"	Blue
Thionin	Purple	"	Blue	Blue	Colourless
Mucicarmine ..	Red	Red	Colourless	Colourless	Faint pink
Ciaccio	Occasional very faint pink	—	Pink	Pink	Colourless
Sudan III	—	—	Pink	Pink	Colourless

* Blue in pigmented layer.

In the first five cases there is agreement between the staining reactions of the secretion, the contents of the ducts and the cuticle. With Mallory the secretion changes its staining reaction after it enters the ducts. With thionin and mucicarmine a positive reaction is given by the secretion in the cells and in the ducts through the tissues, but *not* by the contents of the ducts through the chitin or by the cuticle. In the case of mucicarmine the stain is first taken up lightly round the apex of the gland cells and later, when these are in full activity, the entire cells stain darkly.

These positive reactions for mucus agree with Farkas' results but, as shown by the negative staining of the contents of the ducts through the chitin, it by no means follows that the final product of the glands is mucus. Mucus and chitin are closely related substances. They are both glycoproteins, the former yielding galactosamine on hydrolysis, and the latter glucosamine (Mathews, 1921). It is unknown exactly what constituent of mucus gives the purple colour with thionin or the red colour with mucicarmine, and there is certainly no reason why these colour reactions should not be given by chitin—or, in the case of the cuticle, by some "chitin-like" substance which may be even

more closely allied to mucus—during the course of its elaboration from carbohydrate, protein and acid. That the secretion in the glands and in the ducts through the tissues gives positive staining reactions for mucus, so far from being opposed to the view that the glands produce the cuticle, may well prove to be evidence in its favour. The presence of glycogen, noted by Farkas, provides additional confirmatory evidence because carbohydrate is an essential constituent of chitin, mucus and allied glycoproteins.

The presence of adsorbed lipid, so clearly demonstrated by Ciaccio's technique in the cuticle and in the ducts through the chitin, is very difficult to determine in the glands, although occasionally faint traces of colour can be seen. Lipoid does appear, though faintly, rather more often in wandering cells in the tissue round the glands, and it is possible that they may be carrying lipid to the glands. Even in the cuticle the red colour, though general and perfectly distinct, is faint, and it is not therefore surprising that it is difficult to obtain a clear colour reaction from the very small quantities of lipid which are all that can be present in any individual gland cell. It is worthy of note that Paul and Sharpe (1919) found that in *Carcinus*, though the weight of fat in the hepatopancreas increases both in total amount and relatively to the weight of the body and hepatopancreas as ecdysis approaches, *the quantity of phospholipin falls*. They thought that this was converted into nucleoprotein, of which there would be a great demand owing to increased cell division during ecdysis. But it is now known that the apparent sudden growth at each moult is due, not to increase in the number of the cells, but to the taking up of water. It may be that this phospholipin is carried to the tegumental glands and finally appears adsorbed on the substance of the cuticle.

The region of softened chitin between the old and the new integuments can confidently be attributed, as has been shown, to the action of the nuclei which penetrate into it from the tissues. There is therefore no need to postulate, as Farkas has done, that the tegumental glands produce anything in the nature of an exuvial fluid.

Finally sections of the new chitin from the cesophagus of *Palinurus*, fig. 10, Plate 14, provide an interesting demonstration that the secretion from the glands does set as a solid substance after extrusion. In these sections the openings of the glands actually *project (p.d.)* for about $1.5\ \mu$ beyond the surface of the cuticle.

VII. Discussion.

It has been shown in this paper that the integument of the Decapod Crustacea consists of two portions, a thin superficial cuticle and a much thicker under-

lying chitin. The latter in certain regions is sub-divided structurally though not, except in so far as it is calcified, chemically. The cuticle differs from the chitin both structurally and chemically. It does not react in the same way as chitin and is clearly a different substance, its exact nature being as yet unknown. It has a distinct origin, being formed not by the chitogenous epithelium, but by the tegumental glands the function of which has hitherto been a subject for speculation. It is clearly a substance with a low surface tension because, despite the fact that it is produced by groups of glands more numerous in certain areas than in others—there are none, for example, in the stomach—the cuticle forms a homogeneous layer everywhere over the surface of the chitin. It is noteworthy, however, that the cuticle is thickest where the glands are most numerous, in the oesophagus and labrum. The reason for its exceptional thickness there is probably the continual abrasion by hard material, such as the calcified limbs of other Decapods, etc., which is swallowed. Vitzou states that the cuticle, and the outermost, pigmented layer of the chitin, are fully formed before the shell is cast, the deeper, calcified layer of the carapace being formed later and subsequently calcified.

It appears that as the secretion from the tegumental glands is extruded it spreads out as a thin sheet over the surface of the chitin and that this is continually added to until the full thickness of the cuticle is attained. During this process the cuticle is protected by the old integument, the innermost layers of which are softened or dissolved by the action of cells which pass into it from the tissues before and during ecdysis. The secretion from the tegumental glands clearly has the power of dissolving its way through the first formed chitin, the ducts thus formed through this being kept open by the continual flow of secretion through them as the chitin increases in thickness. The cuticle solidifies as a homogeneous layer, though this possibly does not occur until it is exposed when the old integument is shed. This solidification may be the cause of the state of tension in which the cuticle normally exists. The final structure of the integument and of its spines can only be accounted for by an outpouring of the cuticular substance over its surface.

The tegumental glands in the pleopods of the female have, as already stated, been regarded as the source of the cement which binds the eggs to these appendages. Certain preliminary investigations on the nature of this cement support this view, as they indicate that it is of the same nature as the cuticle. It is hoped to deal with this matter fully later. At the present juncture it is only necessary to point out that such an accessory function of the glands is in no way incompatible with their primary function. Since egg laying always

takes place after ecdysis the secretion will not be protected but be discharged directly into the water. Thus it need not necessarily spread over the surface of the integument but may adhere to anything—eggs and appendages—with which it comes in contact, solidifying almost immediately.

The all-importance of the cuticle in controlling the permeability of the uncalcified chitin in Decapod Crustacea, primarily owing to its possession of adsorbed lipid, will be shown in subsequent papers. There appears to be excellent reason for regarding the cuticle as of *fundamental significance* in the life of the Crustacea by insulating them from their environment. Although, owing to its great thickness, particularly easy to demonstrate in the foregut of the Decapods, the cuticle appears to be universally distributed throughout the Crustacea. Ambrohn (1890), by means of the chlorzinc-iodine test, identified two layers in the integument of a variety of Crustacea from Branchiopods to Decapods. The inner layer only gave a positive reaction and this Ambrohn held to be proof that it was of cellulose allied to the tunicin of tunicates; the outer layer—cuticle—he considered to be of chitin. Zander (1897), using the same technique, confirmed the presence of the two layers, only the inner of which gave a positive reaction, in Cladocera, Copepoda, Cirripeda, Mysidacea, Isopoda, Amphipoda, and Decapoda. Unlike Ambrohn, he considered that the inner and not the outer layer was of chitin. This fact was confirmed by Wester (1910) using the technique already outlined, but he failed to find an outer layer at all. The explanation of this has been shown in this paper—it is the destructive action of boiling concentrated alkali on the cuticle.

The presence of the cuticle, if the results of this research are correct, entails the presence of tegumental glands. Glands of this type, with intracellular ducts, have been shown to occur throughout the Crustacea, as the following list, by no means exhaustive, indicates:—

Branchipoda—*Branchipus* (Spangenberg, 1875), *Branchipus* and *Artemia* (Claus, 1886).

Ostracoda.—Various fresh-water species (Claus, 1892).

Copepoda.—Corycæidæ (Haeckel, 1864), *Argulus* (Claus, 1875), various other species (Claus, 1881).

Mysidacea.—Various species (Gelder, 1907).

Tanaidacea.—*Apseudes*, *Tanais* (Claus, 1884, b, 1888).

Isopoda.—*Porcellio* (Huet, 1883), *Asellus* (Ide, 1891), *Anilocra* (Rath, 1895).

Amphipoda.—*Phronima* (Claus, 1872, 1879; Mayer, 1878; Zimmermann,

1898), Corophiidæ (Hoek, 1879), Caprellidæ (Haller, 1880; Mayer, 1882), various species (Nebeski, 1880), *Vibilia* (Ide, 1891), *Ampelisca* (Della Valle, 1893).

Euphausiacea.—Various species (Gelderd, 1907).

Stomatopoda.—*Squilla* (Claus, 1884, a).

Decapoda.—Already cited.

An outstanding confirmation of the very important function played by these glands throughout the life of the Crustacea is afforded by Allen's statement that they are present and well-developed in the day-old larvæ of *Palæmontes*. In certain Amphipoda, e.g., *Ampelisca*, which form nests the glands may have an accessory function in the formation of the threads of which these are composed. In the Cirripedia the secretion of the cement gland may prove to be similar to the cuticle.

A similar state of affairs appears to be widespread, if not universal, in the other Arthropoda. By chemical tests both Ambronn and Zander identified two layers in the integument of various Insecta, Arachnida (including *Limulus*) and Myriapoda. Structurally the integument of the Insecta can be divided into a homogeneous outer layer and a thicker, lamellated inner layer (Holmgren, 1902; Plotnikow, 1904), the inner of which may be further subdivided (Lécaillon, 1907; Kapzov, 1911) as it is in the carapace of the Decapods. Campbell (1929) speaks of an extremely thin, superficial epicuticula (Grenzlamelle), a brittle, pigmented exocuticula (Pigmentschichte) and a flexible, colourless endocuticula (Hauptlage). Schulze (1913) and Hass (1914) have called the first of these the "Sekretschichte" (though they failed to find the source of the secretion) and pointed to the morphological and chemical distinctions between this and the pigmented exocuticula. The exocuticula and the endocuticula certainly contain chitin, but according to Kühnelt (1928, a and b)* the epicuticula does *not*, but fatty acids and cholesterol occur in it while it has many properties in common with the cuticle of plants. Dermal glands are widespread throughout the Insecta (Imms, 1925), examples being those in the larvæ of the Diptera (Keilin, 1913) and the glands of Verson in larval Lepidoptera which Buddenbrock (1930) thinks are not responsible for the exuvial fluid which, in his opinion, comes from the epithelial cells. This raises the question of the true function of the exuvial glands throughout

* These papers, not seen till after this paper had gone to the press, are of special value, showing that the "Grenzlamelle" of the Insecta has many properties in common with those, here described, of the cuticle of the Decapod Crustacea.

the Insecta. In the Myriapoda, Fuhrmann (1921) has described tegumental glands of the same general type and in his figure shows the integument with a distinct superficial layer. In *Limulus*, Patten (1894) describes and figures tegumental glands (which he regarded as sense organs) of almost exactly the same type as those of the Decapods.

The above very far from exhausts the literature on the structure of the integument and on the presence of dermal glands in the Arthropoda, but enough has been quoted to indicate that the state of affairs found to exist in the Decapod Crustacea may be of universal occurrence throughout the Arthropoda. Finally the work of Sanford (1918) and of Abbott (1926) on the permeability of the chitin lining the crop of the Blattidæ indicates that here too there is an adsorbed lipid.

VIII. Summary.

(1) The integument of the Decapod Crustacea consists of two distinct layers, a thin superficial cuticle which is hyaline and a much thicker underlying chitin which is lamellated and may be subdivided into various regions.

(2) The cuticle is further distinguished from the chitin by its staining reactions, iso-electric point, behaviour in boiling concentrated alkali and in cold concentrated mineral acids and Schulze's mixture resistance to the action of chitinase from *Helix*, possession of adsorbed lipid, and by the state of tension in which it normally exists. The cuticle is *not* chitin.

(3) The contents of the ducts which traverse the chitin from the tegumental glands have the same properties as the cuticle.

(4) The structure of the tegumental glands and of their intracellular ducts are described. These occur everywhere beneath the chitin but are most numerous in the labrum and anterior half of the cesophagus.

(5) The unsatisfactory nature of previous theories as to the function of these glands is shown.

(6) The degree of formation of the gastroliths has been used as an indication of the preliminary stages of ecdysis.

(7) Before and during ecdysis the undermost layers of chitin are softened and possibly dissolved by the action of cells which migrate into them from the tissues.

(8) New chitin begins to appear when the gastroliths attain medium size. The cuticle appears a little later and then the two increase in thickness.

(9) The chitin is formed by the chitogenous epithelium, but the substance of the cuticle is elaborated within the tegumental glands and then flows over

the surface of the chitin as a thin film which increases in thickness and solidifies, possibly as a result of exposure after ecdysis, when fully formed.

(10) The tegumental glands exhibit greatest signs of activity when the formation of the cuticle is at its height, *i.e.*, when the gastroliths are large. After functioning they degenerate and new glands are formed immediately. At no other period does degeneration and formation of new glands occur.

(11) It is difficult to determine the presence of lipid in the secretion in the glands, but both the presence there of glycogen and of substances giving a positive staining reaction for mucus are evidence that a "chitin-like" substance is elaborated.

(12) During its formation the openings of the ducts may project above the surface of the cuticle, affording additional evidence that the secretion forms the cuticle.

(13) The spreading out of the secretion as a thin continuous layer is probably explained by low surface tension.

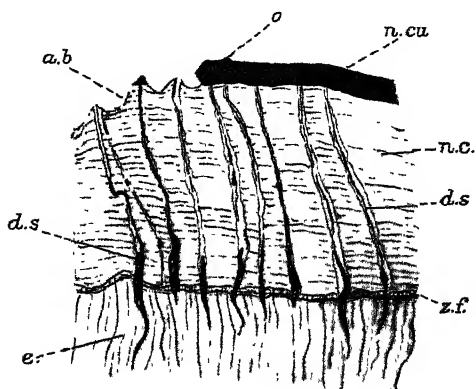
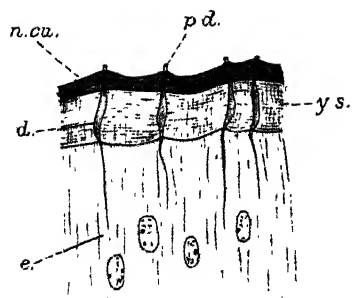
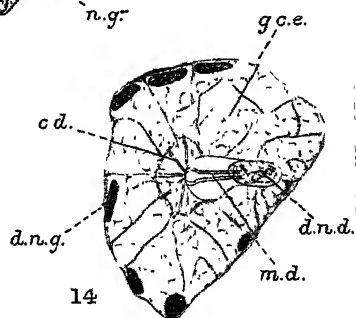
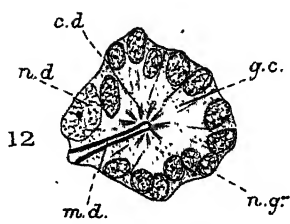
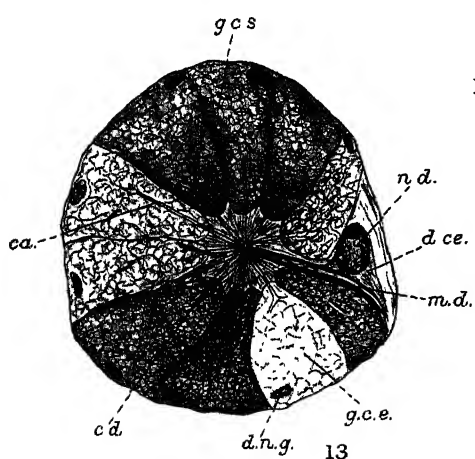
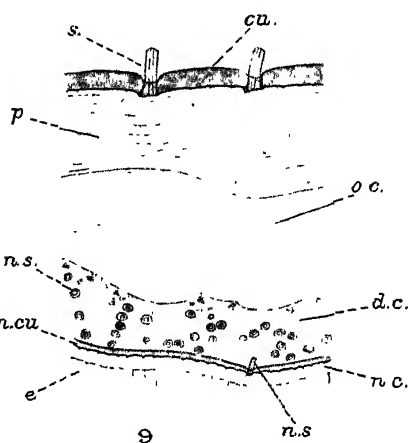
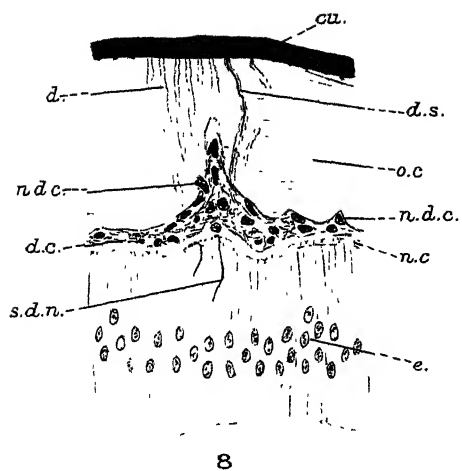
(14) The view that the tegumental glands on the pleopods of the female produce the cement which binds the eggs to these appendages is probably correct. This accessory function of the glands is entirely compatible with their primary function.

(15) The presence of the cuticle and of tegumental glands appears to be universal throughout the Crustacea, and in very many, if not all, other Arthropoda. It is suggested that the function of the dermal and the so-called exuvial glands in larval Insecta requires re-examination in view of the present results.

(16) The cuticle is probably of fundamental importance in controlling the permeability of the uncalcified integument in Decapod and other Crustacea, and possibly in all Arthropoda.

IX. DESCRIPTION OF PLATE 14.

Lettering.—*a.b.*, area of chitin from which cuticle has been torn off in sectioning; *c.d.*, collecting ducts; *ca.*, capillary ducts; *cu.*, cuticle; *d.*, ducts of tegumental glands through chitin; *d.c.*, region of dissolving chitin; *d.ce.*, duct cell; *d.n.d.*, degenerating nucleus of duct cell; *d.n.g.*, degenerating nucleus of gland cell; *d.s.*, duct containing secretion; *e.*, chitogenous epithelium; *g.c.*, gland cell; *g.c.e.*, gland cell empty after secretion; *g.c.s.*, gland cell full of secretion; *m.d.*, main duct; *n.c.*, new chitin; *n.cu.*, new cuticle; *n.d.*, nucleus of duct cell; *n.d.c.*, nuclei in region of dissolving chitin; *n.g.*, nucleus of gland cell; *n.s.*, spine on new chitin; *o.*, opening of duct from tegumental gland on surface of cuticle; *o.c.*, old chitin; *p.*, pigmented layer of chitin; *p.d.*, projection of opening of ducts above surface of new chitin; *s.*, spine; *s.d.n.*, secretion in ducts from tegumental glands beginning to pour over surface of new chitin; *y.s.*, chitin showing vertical as well as horizontal striations; *z.f.*, zone of formation of chitin by epithelium.



- FIG. 8.—*Homarus*, transverse section through anterior region of œsophagus from animal with medium sized gastroliths. Fixed Bouin, stained iron hæmatoxylin. 6 μ . \times 265.
- FIG. 9.—*Homarus*, transverse section through chitin and edge of epithelium of labrum from animal with large gastroliths. Fixed and stained by Ciaccio's lipid method. 6 μ . \times 265.
- FIG. 10.—*Palinurus*, transverse section through new chitin and portion of epithelium from animal about to moult, old chitin not shown. Fixed Flemming, stained iron hæmatoxylin. 8 μ . \times 600.
- FIG. 11.—*Homarus*, transverse section through anterior region of œsophagus from animal with very large gastroliths. Old chitin stripped off in fixation. Fixed Bouin, stained Mallory's connective tissue stain. 6 μ . \times 600.
- FIG. 12.—*Homarus*, section through centre of a newly formed tegumental gland from animal with large gastroliths. Fixed Bouin, stained Delafield's hæmatoxylin. 6 μ . \times 600.
- FIG. 13.—*Homarus*, section through centre of a mature tegumental gland in state of active secretion, from animal with very large gastroliths. Fixed Bouin, stained Mallory. 6 μ . \times 600.
- FIG. 14.—*Homarus*, section through tegumental gland which is degenerating after discharging its secretion, from animal with very large gastroliths. Fixed Bouin, stained Mallory. 6 μ . \times 600.

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The Cultivation of Vaccinia Virus: A New Series of Subcultures in Cell-Free Medium.

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It is now generally accepted that vaccinia virus may be cultivated in a medium containing living tissue and that cell proliferation is not essential for multiplication of the virus. Also it is stated that survival and increase of virus cannot occur, except in the presence of living cells, for any appreciable period at incubator temperature. Although the earlier literature presents several accounts of successful cultivation on lifeless media, these have not withstood the criticism which improved methods of testing for the presence of virus have brought to bear on them. After considerable experience in cultivating vaccinia virus both by tissue culture and on the rabbit kidney medium used by Maitland and Laing (1930), a short series of experiments was reported demonstrating the possibility of cultivating the virus in an apparently cell-free medium (Eagles and McClean, 1930). Further evidence substantiating these results and demonstrating considerable multiplication in cell-free media was presented by Eagles and McClean (1931). Other investigators, notably Rivers (1931) and Maitland, Laing and Lyth (1932) have so far been unable to secure similar results.

In view of the fundamental importance of cultivating a virus in a lifeless medium, the failure of these later investigators to confirm our results, and in view of the criticism that the kidney extract medium may, in our case, have contained some residual cells or cell-fragments, a new series of experiments was undertaken. The method of preparing the culture medium has been altered with a view to securing one richer in cell substance while rendering the presence of viable cells remote. It has been suggested that the method of calculating the total amount of increase in virus through a series of subcultures by comparing unincubated control and culture at each step may give an erroneous idea of multiplication and that a more precise method might be adopted. Rabbits are known to vary considerably in their response to vaccinia virus. This, however, does not satisfactorily explain the large and unexpected reduction in titre which has occasionally occurred when testing the virus content of the unincubated control. In the series here presented the method of calculating increase of virus through a series of passages has been based on the theoretical potency of a seeding representing in all cases a 1-50 dilution of the previous subculture, which had been experimentally titrated to the end point.

Experimental Methods.

(1) *Preparation of Cell-free Medium.*—A rabbit was bled by cardiac puncture under ether anæsthetic and the blood defibrinated under such conditions as to ensure sterility. The animal was killed with ether, the kidneys removed aseptically through the dorsal route, stripped of fat and capsule and as much as possible of the pelvis removed. In a tissue-culture box the kidneys were minced as finely as possible with curved surgical scissors and ground in a mortar without sand or other material until a smooth, creamy consistency was obtained. 2 c.c. hypertonic saline (9 per cent.) was added and grinding continued until thoroughly mixed. Throughout eight subcultures two sets of experiments were done in parallel. The kidney emulsion was divided into two equal portions in centrifuge tubes. One was allowed to stand for at least 1 hour at room temperature in contact with hypertonic saline and the other, packed in cotton-wool, was placed in a frigidaire for 1 hour at -12° to -13° C., where it was slowly frozen solid. When freezing was complete the tube was plunged into water at about 80° C. and agitated until the contents were completely thawed, a process which rarely required longer than 2 minutes, after which the tube was removed from the water. The slow freezing and rapid thawing was carried out with the object of further disintegrating the cells. Sufficient sterile distilled water to restore normal saline concentration was added to each portion

and thoroughly mixed. The tubes were centrifuged at about 4,000 revolutions per minute for 1 hour. The supernatant fluid was removed, care being taken not to disturb either the surface fatty substance or the underlying cells, and re-centrifuged at the same speed for 1 hour. This second portion of supernatant fluid was removed with similar precautions and used as the basis of the cell-free medium. Stained preparations showed an amorphous deposit and possibly occasional small cell fragments, though these could not be recognised with certainty. Whole cells were not found.

Equal parts of fresh rabbit serum and Tyrode's solution were added to the kidney extract to make a dilution of 1 in 50 of the virus seeding.

Hanging drop preparations and stained specimens of the diluted extract showed the presence of fat, occasional red blood cells from the rabbit serum and, rarely, possible cell fragments.

(2) *Cultivation of the Virus.*—Two sets of experiments were made in parallel, viz., (1) with the extract from the ground kidney treated with hypertonic saline, and (2) with the extract which had, in addition, been frozen and thawed. The other culture conditions were identical.

A fresh testicular strain of neuro-vaccine was diluted 1 in 50 in the unfrozen medium, thoroughly mixed and distributed in 2 c.c. amounts in Carrel and Rivers Flasks. These were kept in a moist incubator at 37° C. for periods varying from 3 to 6 days. Two flasks of each experiment were removed each day during that time and tested in appropriate saline dilutions intradermally in rabbits. One of these cultures, showing a titre of 1 in 100,000, was selected for subculture. Throughout the series the same technique was followed, i.e., the flask showing the highest titre in each culture was diluted 1 in 50 in fresh medium. All flasks were placed in the incubator. Titration of an incubated control was not made. Ten subcultures of the original culture were made in direct series in the unfrozen extract and eight in the frozen and thawed extract. When, as sometimes happened, the virus in all flasks of a set of cultures failed to survive, that particular subculture was repeated.

(3) *Sterility Tests.*—All cultures were tested for sterility at the time of titration by seeding several drops in Hartley's broth. After 3–4 days' incubation subcultures were made on plain agar and retained for a week before discarding.

(4) *Animal Titration.*—Rabbits used for titration were prepared several hours before inoculation. 0.2 c.c. of each dilution of culture material was injected intradermally. At the same time an intradermal injection of 0.2 c.c. of a 1 in 1,000 dilution of lymph used in ordinary vaccination practice was made

to test the sensitivity of the rabbit to vaccinia. Readings were made daily from the third to the sixth day. All animals reacting atypically to the lymph injection were discarded. In our experience rabbits which react typically toward lymph do so toward culture virus. On the other hand a culture which has been negative when tested on an animal that reacted indifferently toward lymph has become positive on re-titration on a vaccinia-sensitive one.

Measurement of Increase in Virus during Subculture.

The original culture, from which 10 subcultures in unfrozen and 8 in frozen material were made in series, gave a titration of 1 in 100,000. The theoretical titre of the virus before incubation has been assumed to be 1/50 of the titre of the inoculum at each subculture. This theoretical titre compared with the highest titre obtained after incubation gave the amount of increase in virus at each subculture. Dilution of the virus of 1 in 50 at each step remained constant.

Results.

Table I shows the behaviour of the virus through 10 generations of subculture in centrifuged kidney extract after treatment with hypertonic saline but unfrozen. Each subculture contains the amount of virus in the one preceding it diluted 1 in 50. The theoretical titre in each case is calculated in terms of dilution and not determined by animal inoculation of the unincubated control. With the exception of the third and seventh subculture, where larger increases

Table I.—Cultivation of Vaccinia Virus in Centrifuged Cell-free Extract after Treatment with Hypertonic Saline.

No. of sub-culture.	Date.	Actual titre before dilution.	Dilution.	Theoretical titre before incubation.	Titre after incubation.	Increase in every subculture.	Actual end increase.	Log of increase.
—	2.10.31	—	—	—	100,000	—	—	0
1	9.10.31	100,000	1 in 50	2,000	100,000	50	50	1.7
2	16.10.31	100,000	"	2,000	100,000	50	50 ²	3.4
3	6.11.31	100,000	"	2,000	1,000,000	500	50 ³ × 10	6.1
4	16.11.31	1,000,000	"	20,000	1,000,000	50	50 ⁴ × 10	7.8
5	27.11.31	1,000,000	"	20,000	1,000,000	50	50 ⁵ × 10	9.5
6	3.12.31	1,000,000	"	20,000	1,000,000	50	50 ⁶ × 10	11.2
7	18.12.31	1,000,000	"	20,000	100,000,000	5,000	50 ⁷ × 10 ³	14.9
8	30.12.31	100,000,000	"	2,000,000	100,000,000	50	50 ⁸ × 10 ³	16.6
9	7.1.32	100,000,000	"	2,000,000	100,000,000	50	50 ⁹ × 10 ³	18.3
10	20.1.32	100,000,000	"	2,000,000	100,000,000	50	50 ¹⁰ × 10 ³	20.0

Total amount of dilution after 10 subcultures = 10¹⁷.

Total amount of increase after 10 subcultures = 10²⁰.

in virus were obtained, the increase in each subculture was equal to the amount of dilution. The tenth subculture shows a multiplication of 10^{20} times the original culture. During the course of subculturing the original culture was diluted 10^{17} times.

This tendency of the virus to show a small but regular increase through a series of subcultures is further illustrated in fig. 1. The logarithm of the greatest increase obtained in each subculture is plotted in the ordinate and the number

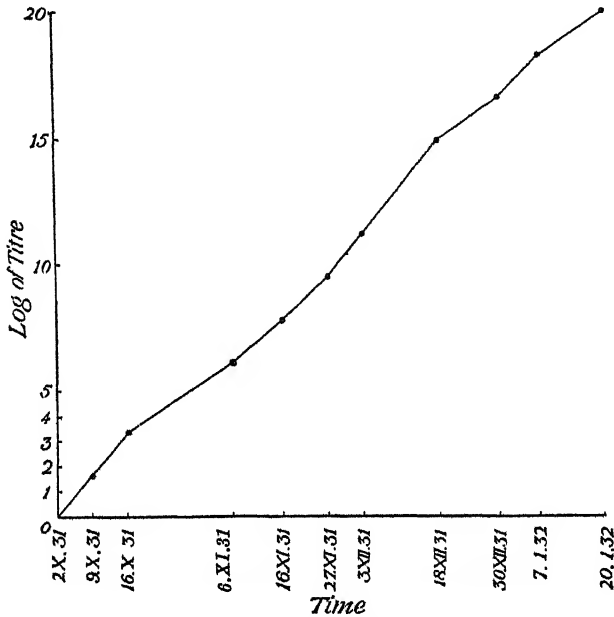


FIG. 1.—Growth of *Vaccinia Virus* in Cell-free Medium.

of subcultures, designated by the date on which they were made, in the abscissa.

Table II shows the results when the hypertonic saline-treated extract was frozen and thawed before inoculation with the virus. Other culture conditions were identical. When the individual flasks in a subculture in the frozen extract were compared with those in the unfrozen extract greater irregularity in increase occurred in the frozen material, as evidenced by the amount of increase in successful cultures and by the number of flasks in any experiment which showed increase.

Examination of Culture Virus for Elementary Bodies.

Nauck and Paschen (1932) have shown that under culture conditions an increase in the number of Paschen bodies present is co-incidental with virus

Table II.—Cultivation of Vaccinia Virus in Centrifuged Cell-free Extract after Treatment with Hypertonic Saline and Frozen and Thawed.

No. of subculture.	Date.	Actual titre before dilution.	Dilution.	Theoretical titre before incubation.	Titre after incubation.	Increase in every subculture.	Actual end increase.
—	16.10.31	—	—	—	100,000	—	—
1	6.11.31	100,000	1 in 50	2,000	1,000,000	500	50×10
2	16.11.31	1,000,000	„	20,000	1,000,000	50	$50^2 \times 10$
3	27.11.31	1,000,000	„	20,000	100,000	5	50^3
4	3.12.31	100,000	„	2,000	100,000	50	50^4
5	18.12.31	100,000	„	2,000	100,000	50	50^5
6	30.12.31	100,000	„	2,000	100,000,000	50,000	$50^6 \times 10^3$
7	7.1.32	100,000,000	„	2,000,000	100,000,000	50	$50^7 \times 10^3$
8	20.1.32	100,000,000	„	2,000,000	100,000,000	50	$50^8 \times 10^3$

Total amount of dilution after 8 subcultures = 5×10^{13} .

Total amount of increase after 8 subcultures = 5×10^{16} .

increase as shown by animal tests. In a recent publication (Eagles and Ledingham, 1932) experimental evidence was adduced which pointed strongly to the probability that the Paschen body represents the active infective agent in a potent filtrate of the virus. Moreover, these bodies were found in large numbers in a culture in cell-free medium after it had been treated in the manner described by Ledingham (1931, 1932) for freeing these bodies from the surrounding medium. In the present series samples were examined from the fourth and eighth subcultures. Elementary bodies were present in large numbers. In cultures in which multiplication did not take place only occasional bodies could be demonstrated. Uninoculated culture material was entirely free of Paschen bodies.

Discussion.

The ability of vaccinia virus to multiply under artificial culture conditions is no longer seriously questioned. Evidence of its ability to do so when living cells are excluded from the medium has been the subject of criticism, in view of failures reported by other workers (Rivers, 1931, and Maitland, Laing and Lyth, 1932). Very little is known concerning the growth requirements of viruses generally, but it is broadly stated that the virus multiplies only in the presence of living susceptible cells and gradually deteriorates when these are no longer viable. It should be stated, however, that broad generalisations of this character are definitely premature in view of the fact that cultivation *in vitro* of vaccinia virus and of viruses generally has hitherto been investigated

by comparatively few workers and by still fewer in an intensive fashion. Maitland, Laing and Lyth suggest that success or failure in virus cultivation is dependent upon a number of factors, the most significant of which are the respiratory activity of cells, which varies considerably in tissues from different organs, and the free access of oxygen to these tissues. They also point out that a proper ionic composition of the saline constituents of the medium is essential for growth, since cells fail to survive in an unbalanced medium.

The accepted fact that favourable conditions in a medium used to cultivate vaccinia virus are those which prolong survival of cells is difficult to reconcile with the results obtained in the course of this work with a cell-free medium. It has been suggested that similar results in our earlier work (Eagles and McClean, 1930) were due to cell fragments being included in the medium. Such fragments may have been present in hanging drop and smear preparation but were difficult to define with certainty. In view of the finding of Rivers, Haagen and Muckenfuss (1929) that cells inside pieces of tissue in culture medium remained alive after 5 days' incubation it is necessary to stress the fact that whole cells were not present in our medium. It is unlikely that any fragments that may have been present remain alive for any significant period. Furthermore, if cells are essential for growth of virus an occasional fragment must fulfil that requirement. When our results are considered it would appear that the essential growth factor is released when the cells are broken up, and that whole living cells are not necessary. The medium must be used fresh. When it is allowed to remain in the cold room or at room temperature for a week it is no longer capable of promoting growth of virus. It does, however, withstand one period of freezing and thawing.

Typical vaccinia lesions, which were neutralised by vaccinia immune serum, were obtained in animals inoculated with virus cultivated in cell-free medium. The nature of these lesions was further proved by the demonstration of large numbers of Paschen bodies in the third and eighth subcultures. If multiplication had not occurred this would have been impossible, especially in the eighth subculture, on account of the enormous dilution of the original virus seeding.

Animal titrations of unincubated controls were not carried out in this series. In former experiments this was done since it was thought that comparison of titrations before and after incubation gave considerably more information regarding the behaviour of the virus throughout the series. The controls occasionally gave a titre considerably lower than expected after dilution in each subculture. When this was followed by a large increase after

incubation an appreciable error in total multiplication was introduced. In the present series any error arising from such comparison does not appear. Maitland, Laing and Lyth found no evidence that vaccinia virus adapted itself to cell-free conditions. In their hands a number of initial attempts failed to establish the virus in culture. In our experience, in the experiments here recorded and in former series, considerable difficulty was encountered in obtaining a successful initial culture, both with tissue-containing and cell-free media. Moreover, it must not be assumed that a successful initial culture assures a successful series of subcultures; frequently subcultures must be repeated. It is not unusual to find that the virus may appear to deteriorate or merely to survive in a subculture but be followed in the succeeding one by a sudden increase giving quite typical reactions.

We have found that the tendency toward irregularity in growth is greater in cell-free than in tissue-containing media. It is well known that samples of any nutrient medium vary considerably in their growth-promoting qualities though the method of preparation is unchanged. Much of the irregularity with cell-free media might be accounted for in this way since the medium must be used fresh. Until the growth requirements of vaccinia virus are more clearly understood than they are at present, and a medium devised whose constituents are consistent, it is unlikely that irregularity in growth in any medium will be overcome. In our experience the presence of fresh tissue, even under apparently the most favourable conditions, does not overcome this difficulty. With improved methods of liberating from cells the maximum amount of the essential substances, cell-free medium should become as satisfactory as cell-containing media for cultivation of vaccinia virus.

Conclusions.

Vaccinia virus has been cultivated through ten generations in subculture in a medium in which whole cells could not be demonstrated. A total multiplication of 10^{20} was obtained. The end point of a titration of the tenth subculture was greater than that of the original culture, although during the course of subculture the original virus seeding had been diluted 10^{17} times. Such a condition could not be accounted for by mere survival especially in view of the amount of dilution and the long periods of incubation at 37° C. entailed in the process of subculturing.

A series of eight subcultures were carried out in the same medium, which had been frozen and thawed. Although freezing does not damage the medium

to any great extent the increases in virus obtained thereafter are more irregular. It is suggested that substances are released from the cells during the preparation of the medium which are, in the fresh state, essential to growth of virus.

The virus is not altered by cultivation in cell-free medium as evidenced by the type of reaction produced in animals, by neutralisation with antiviral serum, and by the presence of masses of elementary bodies in passage subcultures.

The authors wish to thank Professor J. C. G. Ledingham, F.R.S., for examination of cultures for Paschen bodies and also Professor M. Greenwood, F.R.S., Department of Epidemiology, School of Hygiene and Tropical Medicine, for his kind advice regarding the method employed in determining the amount of increase in virus.

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Researches on Plant Respiration. I.—The Course of Respiration of Lathyrus odoratus during Germination of the Seed and the Early Development of the Seedling.

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Introduction.

There is no necessity at the present time to emphasise the fundamental importance of respiration in the life of the plant, since it has been realised for a very long time that this process continues in every actively living cell. Although it is recognised that respiration in its widest sense is to be regarded as a release of energy which is in part utilised in other plant processes, yet the respiratory function is in fact not at all clearly understood, and its mechanism, in spite of plausible theories, remains to a great extent obscure. This obscurity can only be cleared away by the accumulation of quantitative data bearing on the respiratory process. Although a considerable amount of work has been published embodying the results of experimental researches on respiration, only a small proportion of these, when subjected to critical examination, are sufficiently exact to be of real value.

The primary object of the researches to be recorded in this series of papers is therefore the accumulation of quantitative data concerning the respiratory function. At the moment the schemes of Neuberg, Meyerhof and Blackman afford a satisfactory and, on the whole, adequate working hypothesis of the stages of the respiratory process. They rest, however, on a slender basis of experimental evidence albeit of a very satisfactory kind, and a great deal of work is needed either firmly to establish them or to indicate where, and in what respects, they require modification and amplification.

In this first paper we deal with the course of respiration during germination of the seed and the early development of the seedling of the sweet pea (*Lathyrus odoratus*, Linn.). A certain amount of work has already been published on the course of respiration of germinating seeds, notably those of wheat (*Triticum vulgare*), the edible pea (*Pisum sativum*), the broad bean (*Vicia faba*) and cress (*Lepidium sativum*). Much of this work, though not all, can be subjected to criticism on two distinct points. In the first place the measurements in many cases were made at temperatures very imperfectly controlled. It is now well

known that temperature influences respiration rate very considerably, a temperature coefficient of about 2.5 being common between 0° and 30° C., while above this latter temperature the matter is generally complicated by the operation of a time factor. In the second place, owing to the small size of most seeds, and the methods that have been available for measuring respiration, it has generally been necessary to use a considerable number of seeds as the unit for actual measurements of respiratory intensity. This is particularly unfortunate for it is well known that seeds which are apparently alike in constitution and which are subjected to the same external conditions do not necessarily germinate at the same rate. However much care is taken to reduce differences in germination rate of the different members of a sample of seeds, it is practically inevitable that the measured respiration rate of such a sample is the sum of the respiration rates of a number of seeds in different stages of development. This may lead to erroneous conclusions regarding the actual course of respiration, as sudden or fairly sudden changes in respiratory activity taking place at one particular stage in development may be completely masked.

For this reason the method adopted by Blackman and Parija (1928) in their studies on the respiration of apples, in which the course of respiration of the *single individual* is followed, is most desirable in investigations on the course of respiration of germinating seeds. The difficulty, as indicated above, lies in measuring with sufficient accuracy the intensity of respiration of a single seed or seedling. Until recently this difficulty, for all except large seeds, was insuperable. Now, however, the development of the katharometer for work on respiration has placed in our hands an ideal instrument for measuring the respiration of single seeds, and accordingly in all but one or two of the experiments which are included in this first research the unit in each experiment was a single seed.

We propose to investigate the respiration of seeds of various types, such as those storing starch, fat and protein respectively. In the present study we have selected one of the first type belonging to the family Leguminosæ. In previous experimental researches on the respiration of this type of seed the favourite material has been the edible pea, *Pisum sativum*. For our first experiments we have chosen a different species, the sweet pea, *Lathyrus odoratus*, as the seeds of this are less susceptible to invasion by fungi and bacteria than those of the edible pea, they are of a convenient size, and pure strains can readily be obtained. The variety we have chiefly used is "What Joy," the seeds of which germinate readily without a preliminary chipping of the testa. Three samples of seeds were used, the first two being obtained from

Messrs. Simpson of Birmingham, and belonging to the 1930 and 1931 crops respectively, while the third was obtained from Messrs. Sutton & Sons of Reading, and belonged to the 1931 crop.

Method.

Throughout the present work, the respiration of the seeds as indicated by their rate of carbon dioxide output was measured by means of the katharometer. The katharometer, as adapted by the present writers for the measurement of respiration, is described fully elsewhere (Stiles and Leach, 1931) and the instrument used for these researches, together with its plant chamber, is shown in fig. 3 of the above-mentioned account. Further details of experimental methods and apparatus used by us have already been published by one of us (Leach, 1932), and to this account also would we refer our readers.

Although some of the respiration measurements used in this paper were made by taking visual readings of the indicating galvanometer after carefully adjusting the katharometer heating current and noting the galvanometer zero, the majority were obtained by means of an automatic recording device. This apparatus (Leach, 1932) records, photographically, hourly galvanometer readings on strips of bromide paper, and the individual records obtained cover periods extending from 2 to 48 hours. The rates at which carbon dioxide was evolved by the experimental seeds during germination were determined by subsequent measurement of the records.

In the case of a number of seeds, both the oxygen intake and the carbon dioxide output were determined simultaneously. The oxygen intake data were obtained by measuring the change in pressure of the gas in the plant chamber by means of an automatically recording manometer (Leach, 1932), while the carbon dioxide output during germination was being recorded by the katharometer.

Calibration of the apparatus was carried out, in the case of the katharometer, by adding measured volumes of pure carbon dioxide to the air in the plant chamber and noting the resulting change in galvanometer deflexion. This method of calibration assumes that oxygen and air have the same thermal conductivity. We have previously given reasons for supposing that the difference between the thermal conductivities of these gases is negligible, but if, as is sometimes supposed, the conductivity of oxygen differs slightly from that of air, a small correction is necessary in the carbon dioxide values. This is of no importance in the present study as we are not concerned with absolute values of respiration intensity. In the case of the manometer,

calibration was effected by noting the change in galvanometer deflection produced by the addition or withdrawal of measured volumes of air to, or from, the gas already in the plant chamber (Leach, 1932).

It was realised from the beginning that our experimental methods possessed certain sources of error, the chief of which are, a slight falling off in the katharometer heating current during an experiment, shifting of the null point of the galvanometer, and the dissolving of carbon dioxide in the water placed with the seed in the plant chamber at the beginning of an experiment. In the case of the first two these can both be eliminated if visual galvanometer readings are taken, but when automatic recording is resorted to this cannot be done completely without losing the advantages of the method. In practice, however, with well designed and adjusted apparatus the errors arising from these sources have been found to be negligible, those due to current variations being not more than 1 per cent. and those due to movement of the galvanometer null point not more than 0.5 per cent. Both these errors are absent at the beginning of a record, while at the end of the record, which may cover a period of 24 to 48 hours, the first may still be absent, so that the actual error is never more than 1.5 per cent., as both have the effect of making the recorded carbon dioxide output appear slightly larger than it actually is.* With regard to the effect of the water in the plant chamber, this was considered to be too small to warrant any attempt at correction. The difficulties of estimating its exact value with any certainty would be very great and might lead to the introduction of further error instead of elimination of the original one. This error was controlled, however, by always adding the same amount of water, namely, 1 cubic centimetre at the beginning of an experiment.

In the expression of results the evolved carbon dioxide is given as milligrammes of carbon dioxide per gramme of air dry seed, that is, the seeds in the dry state as they were received from the seedsman. Dry weight determinations, for which we are indebted to Dr. W. E. Isaac, show that such seeds contain on an average 9.5 per cent. water. Respiratory quotients are calculated from volumes of carbon dioxide and oxygen reduced to normal temperature and pressure.

The numbers obtained with seeds without testas are not comparable with those obtained for seeds with testas, since the latter are included in the

* The shifting of the null points of the galvanometers has now been practically eliminated by arranging that the electrical circuits of these instruments are only closed for 3 minutes during each hour while recording is taking place.

weights of the intact seeds, but not in the case of those used without testas. The testa comprises about 16.4 per cent. by weight of the whole seed, whence comparison of data obtained with seeds with and without testas can be made by multiplying values obtained with the former by 1.2.

The Carbon Dioxide Output of Intact Seeds during Germination.

As already stated, to secure germination of sweet pea seeds of the variety "What Joy" it is not generally necessary to damage the seed coat. Under suitable conditions of temperature and water supply germination of intact

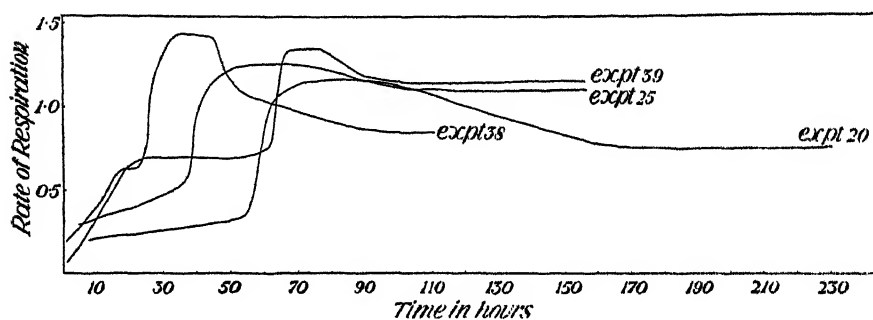


FIG. 1.

seeds takes place readily. In figs. 1 and 2 the course of respiration of a number of these seeds during germination is shown graphically. Each graph shows

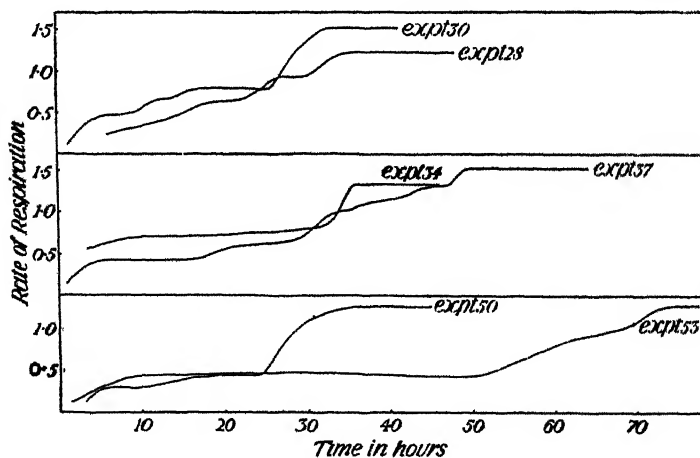


FIG. 2.

the rate of respiration, as measured by carbon dioxide output, of a single seed. Each seed was weighed and then placed dry in the plant chamber together

with a small quantity (1 c.c.) of water or Knop's solution at a temperature of 25° C. The graphs show the course of respiration in the seed from this moment.

Inspection of the figures shows that although the actual course of respiration is not exactly the same in any two cases certain generalisations can be made. When the seeds are brought into contact with water, absorption of the latter takes place and the rate of carbon dioxide output increases rapidly and regularly until a rate of the order of 0.5 mg. carbon dioxide per hour per gram of air dry seed is reached. At this level the rate of respiration remains constant for a shorter or longer period after which it again rises regularly and rapidly until it reaches a value of the order of 1.2 mg. carbon dioxide per hour per gram air dry seed. At this level the respiration rate again remains approximately steady for a number of hours, after which, under the conditions of the experiment, it falls to a somewhat lower level whence any further falling off is so slow that the rate can often be regarded as approximately constant over any 24-hour period. Thus, it will be observed that in experiment 20 the maximum rate was reached after about 90 hours, and that in the subsequent 140 hours the total fall in the rate amounted to about a third of the maximum.

The course of respiration during germination of an intact sweet pea seed and the early development of the seedlings, under the conditions of the experiment, thus goes through five phases : (1) a period of rapidly increasing respiration, (2) a stage of approximately constant respiration rate, (3) a second period of rapidly increasing respiratory activity, (4) a second stage of approximately constant respiratory activity where respiration is at a maximum, (5) a phase of diminishing respiration rate. All five phases are shown by the graphs of experiments 20, 25, 38 and 39 which were continued for periods of 230, 160, 115 and 165 hours respectively. In other cases the last phase is not shown as the experiments were discontinued before the commencement of the phase of slowly decreasing respiration rate. Thus the first four phases are shown by the graphs of experiments 28, 30, 34, 37, 50 and 53 which were continued for 48, 41, 46, 64, 45 and 74 hours respectively.

It will be observed that the length of these phases varies from one individual to another. This is particularly so in the case of the second phase ; thus in experiment 53 it lasted approximately 40 hours, and in experiment 38 only 6 hours.

Exceptionally, this second phase may be omitted. This was the case with experiments 45 and 52, with the result that the maximum respiration rate is

reached in a very short time ; in experiment 45 in less than 18 hours from the commencement of the experiment (*see* fig. 3).

Yet another type of respiration-time curve is shown by experiment 51. Here the respiration rate rises to the constant value characteristic of the second phase and remains there for 50 hours without exhibiting any variation.

The first phase of rapidly increasing respiratory activity corresponds to the period of rapid water absorption by the seed. The absorption of water leads probably both to mobilisation of reserve carbohydrate and to activation of enzymes, and as the concentration of the substrate for respiration or of the

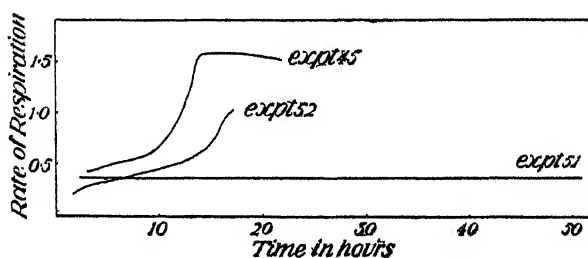


FIG. 3.

enzymes concerned, or both, increase, the rate of respiration increases. This increase in respiration rate continues until the seed is approaching the fully swollen condition. No further appreciable increase in respiration rate then takes place until the splitting of the testa occurs and development of the radicle and plumule proceeds. The length of time required for the splitting of the testa is very variable. In experiment 52, for example, it took place early; consequently we find that the second phase, that of constant respiration rate, is omitted, and the rate of respiration increases continuously from zero to the maximum rate. Such an early splitting of the testa is, however, unusual; more generally, as we have seen, many hours elapse during which the respiration rate is constant, before the splitting of the seed coat allows the embryo to develop, with a concomitant sudden increase in respiration. In experiment 51, where the constant rate continued for 50 hours, it was found that at the end of this time the seed coat was still intact apart from a small hole through which the radicle was slightly protruding.

That this is the correct explanation of the observed course of respiration in the various individual cases is confirmed by observations made on germinating seeds from which the testas have first been removed.

Carbon Dioxide Output of Seeds without Testas during Germination.

The carbon dioxide output of a number of germinating seeds of "What Joy" from which the testas had first been removed are shown in figs. 4 and 5. Inspection of these figures shows that a prolonged second phase, such as is observed in the case of seeds with testas, is not present in the case of seeds from which the coats have been removed. The respiration rate increases continuously, though often not very regularly, from the time the seeds are

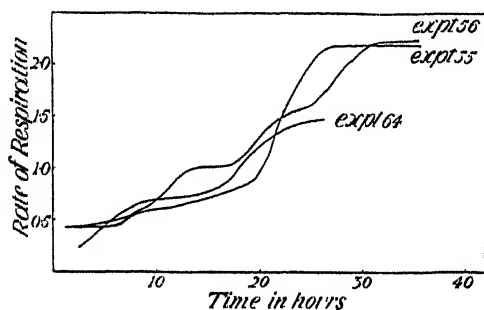


FIG. 4.

brought into contact with water until the maximum respiration rate is reached. In some cases there may be a slowing down in the rate of increase in respiratory activity after the first few hours of water absorption with a subsequent return to a more rapid rate of increase, as in experiments 55, 56, 60 and 64, but this is not always observable, and in no case has the continued respiration at a

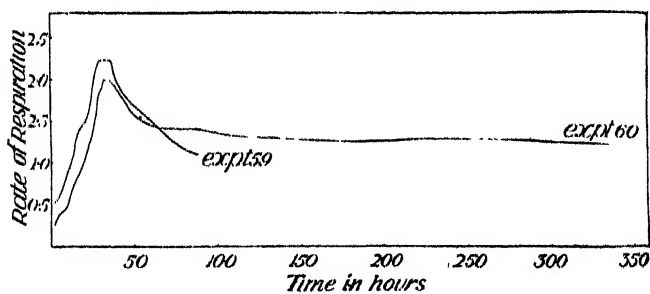


FIG. 5.

constant rate, characteristic of the second phase of respiration of seeds with testas, been observed with seeds from which the testas have been removed. It will also be observed that with these latter there is much less variation in the course of the individual respiration-time curves than is the case with intact seeds, while the maximum rate of respiration is reached after the lapse of a

very much shorter time than is usually the case with intact seeds. The shortening of this time appears to be completely accounted for by the absence of the second phase imposed on the seed by the enclosing seed coat.

The graphs in fig. 5 referring to experiments 59 and 60, which were carried on for 90 and 338 hours respectively, show, under the experimental conditions employed, a comparatively greater and more rapid falling off in respiratory activity after the maximum has been reached, that usually occurs when the testas of the seeds are intact at the beginning. As far as observations go, removal of the testa appears to result in the respiration rate not only reaching its maximum sooner, but in reaching a higher value than would be the case if the testa were retained. Thus the maximum rate in the case of seeds with testas is about 1.2 to 1.4 mg. carbon dioxide per gram of air dry seed per hour. Since the testa comprises about 16.4 per cent. of the whole seed this rate corresponds to one of about 1.44 to 1.68 mg. carbon dioxide per hour per gram of air dry seed *without testa*. This is decidedly lower than the rate observed in the case of seeds which germinated without testas, where the maximum rate is in the neighbourhood of 2.2 mg. carbon dioxide per gram of air dry seed per hour. It may also be of significance that in experiment 45, in which early rupture of the testa took place and in which the course of germination was similar to that observed with seeds without testas, an abnormally high value for the maximum respiration rate was observed, equal to 1.9 mg. carbon dioxide per hour per gram of air dry seed *without testa*.

As indicated in the introduction, the immediate purpose of the researches, of which this is the first, is to obtain quantitative data in regard to the respiration process, our intention being to postpone a general discussion of the results to a later occasion. It may, however, be suggested here that the difference in the value and duration of the maximum observed with seeds provided with testas and those without, may be related to the concentration of respiratory substrate. In the case of seeds without testas the development of the seedling takes place rapidly and the supply of the substrate may be greater at the time of observed maximum respiratory activity than in the case of seeds where respiration has been proceeding regularly for many hours before the testa splits. The more rapid rate of utilisation of substrate in the former case may soon bring about a comparatively rapid fall in substrate concentration with a consequent fall in respiration rate.

In any event we may conclude that the complex form of the respiration-time curves of seeds with testas is due to the presence of the latter. So long as the testa exercises a restriction on development, the respiration rate remains constant.

When this restriction is removed, either artificially or by the bursting of the coat, the rate of respiration continues to increase until the maximum rate is reached.

The Grand Period of Respiration.

Previous investigators on the course of respiration of germinating seeds speak of a "grand period of respiration" during which respiration rises from zero at the commencement of swelling to a maximum after a certain number of days and then falls definitely and regularly. Thus, Mayer (1875), measuring respiration in terms of oxygen evolved, found that the rate of respiration increased to a maximum and then declined, the respiration-time curve being approximately symmetrical on either side of the maximum. The maximum rate was reached in 15 days at 11·8° C. and in 7 days at 23·8° C. Rischavi (1876), who measured carbon dioxide evolution of germinating wheat, obtained a similar result, although the maximum rate of respiration was not reached in his experiments at 21° C. until approximately the twelfth day from the commencement of water absorption. With *Vicia faba*, on the other hand, although he speaks of a grand period of respiration, the carbon dioxide output of his germinating seeds appeared to remain constant, apart from minor fluctuations, from the end of the first to the twentieth day. His experiments with this plant were commenced when the stems were 1 cm. long.

Borodin (1875), who also used carbon dioxide output as a measure of respiration, found that the respiration rate of cress seedlings reached a maximum which was attained on the sixth day at 11–12° C., on the fourth day at 15–16° C., and at the end of the third day at 24° C.

All these researches were carried out nearly 60 years ago, but more recently Fernandes, using more refined methods, obtained an essentially similar result with *Pisum sativum*. At 20° C. both the oxygen absorption and carbon dioxide evolution rose from zero to a maximum in about 3·75 days and then slowly fell, while at 25° C. the course of respiration was similar, but the maximum was reached sooner, namely, in about 2·75 days.

The weight of evidence thus supports the view that on germination respiration of the whole individual increases to a maximum and then falls. This is certainly the conclusion to be drawn with regard to the course of events in wheat, cress, edible pea and sweet pea. The broad bean may be an exception, while as far as they go the observations of Kidd, West and Briggs on respiration of the sunflower suggest that here also there may be no falling off in the respiration of the whole plant after the first few days of development. Thus they found the respiration of a single plant 1, 2, 4, and 13 days old was respectively 0·065,

0.067, 0.073 and 0.282 mg. carbon dioxide per hour. The plants they used were, however, not kept in a darkened plant chamber, and the question arises whether the falling off of respiration rate generally observed to occur after a few days from germination may not be referable to the conditions of the experiment. For example, Fernandes suggested that the decline in respiratory activity might be due to a shortage of mineral salts under experimental conditions.

The observations recorded below shed a certain amount of light on this problem.

Experiment 62.—A single seed weighing 0.0865 gm. without testa was placed to germinate in an incubator at 25° C. After 11 days the main root of the seedling was 3 cm. long and provided with 7 side roots; the stem was 5 cm. long and definitely elongated. The seedling was then transferred to the plant chamber and a record of its respiration rate taken over a period of 7 hours—11 a.m. to 6 p.m.; it respired regularly at the rate of 1.135 mg. carbon dioxide per hour per gram of air dry seed. The seedling was then removed from the plant chamber and placed on the open laboratory bench in front of a window for 24 hours—6.15 p.m. February 13 to 6.10 p.m. February 14. It was then retransferred to the respiration chamber and a record of its respiration rate obtained over a further period. It was found that the respiration rate had now risen to 1.370 mg. carbon dioxide per hour per gram of air dry seed. On the following day the seedling was once more placed on the laboratory bench and left there for more than a day. It was then replaced in the respiration chamber and a record of its respiration rate taken over a further period. For the first 6 hours the respiration rate had risen still higher to 1.725 mg. carbon dioxide per hour per gram of air dry seed, but after this time the rate slowly fell off, but even after 21 hours in the plant chamber the rate had only fallen to 1.480 mg. carbon dioxide per hour per gram of air dry seed, a rate higher than that reached after the first day's exposure to laboratory conditions.

The effect of the change of conditions on the respiration rate is clearly shown in fig. 6.

The rise in respiration rate which results from exposure to ordinary laboratory conditions might be due to a number of causes.

- (1) The rise in respiration rate may be due to the formation of some other substance for which light is necessary, and which furthers some stage in the respiratory process.
- (2) The rise in respiration may be simply due to an increase in the respirable substrate in the shoot by photosynthesis, consequent on the development

of chlorophyll. This, however, does not seem very likely in the case in question as there was no obvious development of chlorophyll during the period of natural illumination through one February day, while the rise in respiration rate is considerable.

- (3) Under conditions in a closed respiration chamber, and to a large extent also in a petri dish in an incubator, transpiration is reduced to a minimum, so that the conduction of respirable carbohydrate from the storage organs to the radicle and plumule by means of the vascular tissue is likely to be slow. Under such conditions, then, we might expect a slowing down in respiration rate as the seedling develops owing to the slowing down of the supply of respirable material to the actively developing regions. If this is so, then transference to an open laboratory bench, either in the light or dark, should lead to an increase in respiration rate by allowing an increase in the supply of carbohydrate to the shoot by means of a more rapid transpiration current.

To test these various possibilities the following experiments were performed.

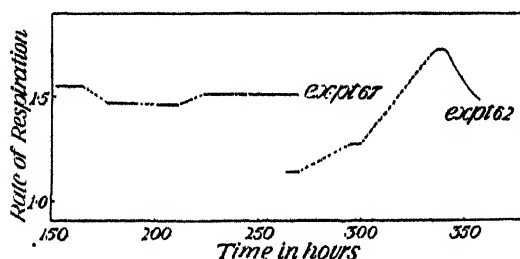


FIG. 6.

Experiment 67.—A single seed weighing 0.0803 gm. without testa was placed to germinate in an incubator at 25° C. After 152 hours the radicle of the seedling was 3.6 cm. long and the stem 1.6 cm. long and yellow in colour. It was then transferred to the plant chamber and a record of its respiration taken over a period of 14 hours (8 p.m. February 19 to 10 a.m. February 20), during which time it was found the seedling respired at the rate of 1.55 mg. carbon dioxide per hour per gram of air dry seed. The seedling was then removed from the plant chamber and transferred to the laboratory bench in the light as in the case of the seedling used in experiment 62, but in a petri dish, whereas in the case of experiment 62 the seedling was uncovered. Here it remained during the day. At 6.20 p.m. the seedling was once more placed in the plant chamber and a record of its respiration taken over a period of 15 hours. The

respiration rate over this period was actually less than before exposure to light, having fallen to 1.475 mg. carbon dioxide per hour per gram of air dry seed. The seedling was then once more exposed to light, but covered by a petri dish (from 10.30 a.m. to 7.30 p.m. February 21). Its rate of respiration after this treatment had not appreciably changed, being now 1.46 mg. carbon dioxide per hour. The same procedure was continued for two more days, but the respiration rate after another day in the light was only 1.51 mg. carbon dioxide per hour, and after yet another day there was no change in the respiration rate. It thus appears that in this case exposure to light did not bring about any increase in respiration rate, the rate remaining practically constant for 5 days. The results are clearly shown graphically in fig. 6.

Experiment 68.—A single seed weighing 0.0726 gm. without testa was placed to germinate on the laboratory bench. When 7 days old the seedling possessed a radicle 3 cm. long and a stem 1 cm. long and was green. A record of its respiration rate was then taken over a period of 7 hours (11 a.m. to 6 p.m. February 20), during which time the seedling respired regularly at a rate of 1.73 mg. carbon dioxide per hour per gram of air dry seed. The seedling was then removed to a petri dish and kept in the dark until 10.30 a.m. on February 21. The respiration rate was once again taken over a period of 8 hours (11 a.m. to 7 p.m. February 21), but had fallen to 1.60 mg. carbon dioxide per hour per gram of air dry seed. Once again the seedling was transferred to a petri dish in the dark until 9.40 a.m. on February 22. The respiration rate was then again measured over a period of 8 hours (10 a.m. to 6 p.m. February 22), and was found to have further fallen to 1.55 mg. carbon dioxide per hour per gram of air dry seed. At the end of this time the plumule was still green, but it appears that in spite of this, maintenance of the seedling in the dark and in a fairly small enclosed space results in a slow fall in the respiration rate as already noted for non-green seedlings, although over a short period of time the change in rate is slight.

Experiment 73.—One seed weighing 0.0620 gm. without testa was put to germinate in a petri dish and kept in the dark for 16 days. The respiration rate of the seedling was then measured over a period of 3 hours and was found to be 1.945 mg. carbon dioxide per hour per gram of air dry seed. The seedling was then placed in a dark room, but without a covering, so that the shoot was exposed to dry air. Here it remained for 21 hours, after which the respiration rate was again measured. As a result of exposure to the air in the dark the rate had risen to 2.561 mg. carbon dioxide per hour per gram of air dry seed, an increase of over 30 per cent.

Experiment 74.—One seed weighing 0.0759 gm. without testa was put to germinate in a petri dish and kept in the dark for 16 days. It had thus exactly the same preliminary treatment as the seedling used in experiment 73. The respiration of the seedling was measured and was found to be 1.676 mg. carbon dioxide per hour per gram of air dry seed. It was then transferred to the laboratory bench in the light without any covering, where it remained for 22.5 hours (5.10 p.m. February 29 to 3.40 p.m. March 1), after which the respiration rate was again measured. It was found that the respiration rate had risen to 2.531 mg. carbon dioxide per hour per gram of air dry seed, an increase of about 50 per cent.

Experiment 75.—One seed weighing 0.0673 gm. without testa was put to germinate in a petri dish in the dark, and kept there for 16 days. The early history of the seedling was thus the same as that of the seedlings used in experiments 73 and 74. A record of its respiration was then taken. Over a period of 12 hours the respiration of the seedling in the plant chamber maintained a constant rate of 1.95 mg. carbon dioxide per hour per gram of air dry seed. For 5 hours after this time the respiration rate showed some falling off, possibly due to the accumulation of carbon dioxide in the plant chamber. The seedling was then returned to the petri dish in the dark where it remained for 9 hours (10.30 a.m. to 7.30 p.m. March 1), after which its respiration rate was measured and found to be 1.74 mg. carbon dioxide per hour per gram of air dry seed. Maintenance of the ordinary experimental conditions, namely, darkness and restriction to a confined space, thus lead to a falling off in respiration rate. This experiment thus confirms the observations already recorded with regard to the course of respiration under such conditions.

Experiment 78.—One seed weighing 0.0750 gm. without testa was put to germinate in a petri dish at 23° C. After 7 days its respiration rate was measured over a period of 4 hours and found to be 1.614 mg. carbon dioxide per hour per gram of air dry seed. It was then transferred to the dark room but with the shoot exposed to dry air, and after 43.5 hours under these conditions its respiration rate was again determined. This was found to have risen to 2.328 mg. carbon dioxide per hour per gram of air dry seed, an increase of 44 per cent. The seedling was then returned to a petri dish in the dark and exposed to a damp atmosphere, where it remained for 3 days. The respiration was then measured once more, and over a period of 3.5 hours was found to be at the rate of 2.048 mg. carbon dioxide per hour per gram of air dry seed, and so showed a definite decline. The seedling was again returned to the dark room, but this time with the shoot exposed to the dry air of the room, and after a further

3 days under these conditions the respiration rate was again measured. It had again increased and was now 2.374 mg. carbon dioxide per hour per gram of air dry seed. Throughout the course of this experiment, therefore, exposure to dry air of the dark room brought about an increase in respiration rate, while exposure to moist air brought about a decrease in respiration rate.

Experiment 80.—A single seed weighing 0.0804 gm. without testa was put to germinate in a petri dish at 23° C. After 7 days a record of the respiration rate was made; the latter was 1.54 mg. carbon dioxide per hour per gram of air dry seed. The seedling was then replaced in the petri dish in the dark and left there for 2 days; the respiration rate after this time had not appreciably changed, being 1.53 mg. carbon dioxide per hour per gram of air dry seed. After a further 3 days under the same conditions the respiration rate had changed little, being then 1.60 mg. carbon dioxide per hour per gram of air dry seed.

From these experiments we may draw the following conclusions:—

- (1) While a seedling is kept in a petri dish in the dark the respiration rate undergoes no appreciable change (experiment 80) or, more usually, slowly declines (experiments 75, 78).
- (2) Transference of a seedling from a petri dish or closed plant chamber either in the light or in the dark to similar conditions in the light brings about practically no change in respiration rate of the seedling (experiments 67, 68).
- (3) Transference of a seedling from a petri dish or closed plant chamber in the dark to the open exposure of a laboratory bench in a light room results in a very marked increase in respiration rate (experiments 62, 74).
- (4) Similarly, transference of a seedling from a petri dish or closed plant chamber in the dark to the open laboratory bench in a dark room also results in a very considerable increase in respiration rate (experiments 73, 78).

Thus, the respiration rate under the conditions afforded by enclosure in a petri dish is considerably depressed below that of respiration in the open, whether in light or in the dark. Two explanations of this behaviour appear possible. In the first place there may be a certain accumulation of carbon dioxide in the atmosphere surrounding the seedling, and this may depress the rate of respiration as shown by Kidd (1915). In the second place the moist atmosphere within a petri dish acts as a powerful depressant of transpiration. In older seedlings the movement of respirable material from the cotyledons to

the young shoot is probably largely brought about by the transpiration stream, so that, under the petri dish conditions, there may be a limited amount of respirable material in the shoot. If this is the case, the increase in respiration rate observed after a time when a seedling is transferred from a closed to an open space is explained by the readier transport of dissolved substances increasing the concentration of respirable material in the apex of the actively growing young shoot.

Although accumulation of carbon dioxide may have a slight effect, we consider the observed results are to be explained mainly on the lines of the second suggestion given above. For the falling off in respiration rate after a maximum is reached was observed by Rischavi (1876) in the case of wheat seedlings 12 days old, and in his experiments the seedlings were kept in a current of moving moist air. Under such a condition, therefore, carbon dioxide cannot accumulate, but a saturated atmosphere, and therefore minimum transpiration rate, is assured.

It does not, however, seem possible to explain in this way the maximum observed as occurring in the case of *Lathyrus odoratus* after about a day, for transpiration can scarcely be operative in so early a stage of development of the seedling. The evidence of this maximum appears rather to be explicable on the lines suggested earlier in this paper, as due to the sudden using up and partial depletion of respiratory substrate at the time of rapid development of radicle and plumule. It is probable that this maximum corresponds with that observed by Borodin (1875) for cress and Fernandes (1923) with the edible pea. We are, however, inclined to think that this maximum was missed by Mayer and Rischavi, partly on account of the variability of the respiration values they obtained, and partly because of the large number of seeds used in one experiment, and that the falling off in respiration they observed after a week or two was largely traceable to experimental conditions inasmuch as they retarded transpiration and therefore the movement of water and dissolved substances.

We have already mentioned that Fernandes suggested that the falling off in respiration rate might be due to a shortage of mineral salts. We have been unable to confirm this suggestion, for the falling off in respiration rate occurs whether the seedling is supplied with water or with Knop's solution.

The Respiratory Quotient.

The respiratory quotient, that is, the ratio of the volume of carbon dioxide evolved to that of oxygen absorbed in respiration, is with reason regarded as of

importance inasmuch as its magnitude may shed light on the nature of the respiratory substrate and on the respiratory process in general. In the case of seeds such as *Lathyrus odoratus* with carbohydrate reserve, it is to be expected that the respiratory quotient would be in the neighbourhood of unity. Nevertheless, existing data are rather contradictory. Thus in the case of germinating wheat, Bonnier and Mangin (1884) found the respiratory quotient at the commencement of germination to be unity or a little higher, but that the quotient fell to about 0.7 after about 2 days after which it gradually rose, approaching unity after another 7 or 8 days. With germinating seeds of *Pisum sativum* they found the quotient to be much less than unity from the beginning. The later results of Fernandes and Frietinger are in sharp contrast with this. The former found that at 20° C. the quotient, from the first to the third hour from the commencement of water absorption, was 3.33, and during the next 2 hours was 2.20, the value thereafter slowly falling until after 30 hours it was about 1.1, round about which value it remained for the next 4 days, the actual determinations varying between 1.17 and 1.01. At 25° C. the behaviour was essentially the same. The quotients found for the same species by Frietinger were still higher, being 4.2 after a period of 16 hours swelling in distilled water, and falling to about 2.8 after 7 hours. Even after a further 24 hours, however, the quotient was found to be still as high as 2.3. Removal of the testa was found to bring about a reduction in the quotient, but even so, a value of 2.4 was found after the 16 hours swelling in water, and after the lapse of a further 30 hours the quotient was still found to be considerably higher than unity, namely, 1.4. Similar high values were also recorded by the same worker for germinating wheat.

We have found no indication whatever of such abnormally high respiratory quotients in the case of the sweet pea, provided the testas are first removed. A number of experiments were carried out in which both carbon dioxide evolution and oxygen absorption of germinating sweet pea seeds were measured as described in the second section of this paper. Usually two or three seeds were used together for these experiments in order to obtain a significant movement of the manometer. It was found that the respiratory quotient of the seeds without testas was never far removed from unity. Thus, in one experiment extending over 43 hours the observed values of the quotient varied between 1.06 and 0.96; in another experiment they varied between 0.99 and 0.95. The observed values are thus usually slightly below unity, but the divergence is so slight and involves such a relatively small movement of the manometer that we are not disposed to lay any emphasis on it since it may be largely within the range of experimental error.

The quotients observed in the case of seeds retaining their testas showed considerable variation and in one case at least exhibited very high values. The conditions of oxygen supply in this case appear undoubtedly to be complex and may necessitate a prolonged investigation for their elucidation.

Summary.

The output of carbon dioxide from germinating seeds and young seedlings of a variety of the sweet pea, *Lathyrus odoratus*, Linn., has been measured by means of the katharometer. By the use of this instrument the course of respiration of individual seeds can be followed. In the case of seeds retaining their testas this course is not simple but exhibits a series of phases corresponding to the various phases of germination. These phases are: (1) a fairly rapid increase in respiration rate as the seeds absorb water, (2) a period characterised by constant respiration rate of very variable duration, which continues until the seed coat is ruptured, (3) a very rapid rise in respiration rate following the rupture of the testas, (4) a period of approximately constant respiration rate when the latter is at a maximum, which is followed by (5) a phase of slowly diminishing respiration rate. Removal of the testas has the effect of largely eliminating phase 2, with the result that respiration rate continually rises to the maximum of phase 4. A number of experiments were performed with the object of finding a reason for the decline in respiration rate during phase 5. The results of these experiments are interpreted as indicating that in older seedlings this decline in respiration rate is due, at least in part, to the conditions of experimentation which tend to a reduction in transpiration rate and so to a reduction in the rate of conveyance of respirable material from the cotyledons to the growing parts, and so to a limitation of the amount of respirable material available at the places where respiration is normally most active.

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The Oxygen Affinity of Chlorocruorin.

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Chlorocruorin is a pigment which is red in concentrated solution and green in dilute solution. The only situation in which chlorocruorin is found is in the blood of Sabellid, Serpulid and Chlorhæmid polychæte worms, where it is in solution in the plasma. It is the only naturally occurring substance which both has similar properties to hæmoglobin and is chemically closely related to the latter. The principal chemical differences between the two pigments are that chlorocruorin has a different porphyrin and a different protein from hæmoglobin (Fox, 1926 ; Warburg and Negelein, 1932).

I have lately studied the oxygen affinity of the specific chlorocruorin of *Spirographis spallanzanii*. Since only very small quantities of blood are obtainable from the worms, it was impossible to study whole blood, but was necessary to work with dilute solutions. A spectrographic technique was therefore adopted, which is possible since the absorption spectra of oxy- and reduced chlorocruorin differ in a similar way to those of oxy- and reduced hæmoglobin. The diluted blood, at definite temperatures and hydrogen ion concentrations, was equilibrated with atmospheres having known pressures of oxygen. After each equilibration the light absorption of the solution was measured at the two wave-lengths in the visible region of the spectrum at which there is the greatest difference between the oxidised and reduced forms of the pigment. From the data thus obtained the relative concentrations of the two forms were calculated.

Blood was obtained from the vessels at the base of the crown of *Spirographis* and from the peri-intestinal sinus. The worms came from Roscoff in Brittany. The blood was diluted in 0·6 M Sørensen's phosphate buffer mixtures the p_H of each of which was determined electrometrically. This particular concentration of phosphates was chosen because it corresponds to the molarity of salts in sea water. After dilution, the blood had a concentration of approximately 6 parts per 1000.

For each experiment about 3 c.c. of the diluted blood was placed in a 300 c.c. glass saturator. At one end of the latter was a tap. To its other end a small

trough, with plane parallel glass faces 2 c.c. apart, was attached by a ground glass joint. The oxygen pressures in the saturator were varied by (a) filling it with pure oxygen at atmospheric pressure, (b) evacuating it completely, (c) filling it with air, and (d) reducing the air pressure by known amounts. The last-mentioned operation was done by connecting a water vacuum-pump to the saturator, with a mercury manometer between the two. After partial evacuation at a known temperature, the oxygen pressure in the saturator is equal to $0.21(b - v - m)$, where b is the barometric pressure, v the vapour pressure of water at the given temperature, and m the manometer reading. The diluted blood was equilibrated with the gas at each oxygen pressure by rotating the saturator horizontally in a water thermostat at the same temperature as that at which the gas pressure had been established. Preliminary trials showed that 15 minutes were necessary for equilibration at 20° C., and 30 minutes at 10° C. To allow for a margin of safety, the saturator was subsequently always rotated in the thermostat for 30 minutes at 20° and 26°, and for 1 hour at 10°.

After each equilibration the saturator was placed vertically, with the trough at its lower end in the course of the light beam of an Yvon spectrophotometer, by means of which the light absorption was then measured at 2 wave-lengths. During these measurements the lower part of the saturator was immersed in a large glass trough with plane parallel faces. This trough contained distilled water at the temperature of the experiment.

In each experiment, lasting 1 day, a single sample of blood was studied at one definite p_H and one temperature. During the course of the day, light absorption measurements were made of the fully oxygenated pigment, of the fully reduced pigment, and of various intermediate states in equilibrium with known oxygen pressures. At the end of each experiment the solution was thrown away, fresh blood being used next day, for chlorocruorin, like hæmoglobin, is unstable in dilute solution. Spectrophotometric measurements were made of the fully oxidised pigment both at the beginning and at the end of each experiment. In cases where the light absorption had changed at the end of the day by an amount greater than the experimental error of measurement, the experiment was rejected, while in cases of small differences the average of two readings was adopted for the fully oxygenated pigment.

The optical method used for determining the relative concentrations of oxidised and reduced chlorocruorin in the diluted blood depends on the following considerations.

The relation between the intensity, I_0 , of a beam of light entering a coloured

solution of concentration c , and the intensity, I , of the light issuing, after having passed through a thickness l , is given by the equation

$$\log_{10} \frac{I_0}{I} = Kcl, \quad (1)$$

K being called the extinction coefficient.

In the case of a solution containing two coloured substances of concentrations c_1, c_2 , having extinction coefficients K_1, K_2 , the following relation holds :

$$\log \frac{I_0}{I} = K_1 c_1 l + K_2 c_2 l,$$

provided that no reaction occurs between the two substances resulting in compounds with different light absorptions.

If the extinction coefficients of the two substances at two different wave-lengths, λ, λ' , have been found to be K_1, K'_1 and K_2, K'_2 , the ratio of their concentrations, c_1/c_2 , in a mixed solution can be found by measuring the light absorption, $\log I_0/I$, of the latter at the two wave-lengths, for

$$\frac{\log \left(\frac{I_0}{I} \right)_\lambda}{\log \left(\frac{I_0}{I} \right)_{\lambda'}} = \frac{K_1 c_1 l + K_2 c_2 l}{K'_1 c_1 l + K'_2 c_2 l} = \phi,$$

whence

$$\frac{c_1}{c_2} = \frac{\phi K'_2 - K_2}{K_1 - \phi K'_1}. \quad (2)$$

In the present work, the two substances in solution were oxychlorocruorin and chlorocruorin. Their extinction coefficients, however, were not known, since their absolute concentrations were unknown. But in each experiment

$\log \frac{I_0}{I} = d$ was determined at each of the two wave-lengths, λ, λ' , for oxychlorocruorin (d_0, d'_0) and for chlorocruorin (d_R, d'_R). From equation (1), d is proportional to K , so that equation (2) becomes

$$\frac{c_0}{c_R} = \frac{\phi d'_R - d_R}{d_0 - \phi d'_0}. \quad (3)$$

Then, in each mixture of oxychlorocruorin and chlorocruorin, the ratio of the concentrations of the two substances was found by determining the light absorption of the solution at each of the two wave-lengths, thus obtaining ϕ , and then making use of equation (3).

The wave-lengths chosen for the measurements were those in the visible region of the spectrum where there is the maximum difference in absorption between oxychlorocruorin and chlorocruorin, namely at the apex of the α -band

and in the trough between the α - and β -bands of oxychlorocruorin (Fox, 1926, fig. 3). A re-determination of these wave-lengths gave $\lambda = 604.5 \mu\mu$, $\lambda' = 580.5 \mu\mu$.

A similar method was used by Hüfner (1900) for estimating the relative quantities of oxyhæmoglobin and hæmoglobin in a mixture of the two. The method, however, is considerably more accurate for chlorocruorin than for hæmoglobin, because the difference between the light absorptions of the oxidised and of the reduced pigment is greater for the former than for the latter (compare Fox (1926), fig. 3, with Vlès (1921), figs. 1 and 3).

The results obtained in the present work are given in Table I and fig. 1.

Table I.—Experimental Results from which the Curves of fig. 1 were constructed. The spectrophotometric measurements ($\log I_0/I$) are given in the order in which they were made in each experiment.

Fig. 1, curve No.	Experi- ment No.	p_H .	Tem- perature.	Oxygen pressure, mm. Hg.*	Log I_0/I .		Per cent. oxy- chlorocruorin.
					$\lambda=604.5 \mu\mu$.	$\lambda'=580.5 \mu\mu$.	
1	19	8.0	10	Vac.	0.654	0.676	0
				Ox.	1.230	0.470	100
				19	1.140	0.499	85
				10	1.060	0.574	62
				6	0.805	0.673	18
				15	1.205	0.555	78.5
				19	1.270	0.527	90.5
2	14	8.2	20	Ox.	0.853	0.270	100
				Vac.	0.408	0.426	0
				26	0.836	0.287	93
				15	0.721	0.331	66.5
				10	0.590	0.369	39.5
				6	0.507	0.420	17
3	21	8.0	20	Vac.	0.795	0.856	0
				Ox.	1.714	0.590	100
				16	1.510	0.777	60.5
				22	1.646	0.621	91
				12	1.205	0.773	40.5
				25	1.635	0.634	86
				15	1.341	0.734	55
4	20	8.0	26	Ox.	0.980	0.400	100
				Vac.	0.556	0.606	0
				18	0.838	0.466	64.5
				27	0.922	0.427	87
				12	0.685	0.512	36.5
				22	0.867	0.463	70.5
5	23	7.7	10	19	0.970	0.551	65
				Vac.	0.500	0.543	0
				Ox.	0.970	0.410	100
				22	0.855	0.461	70.5
				18	0.703	0.501	40

* Ox = atmosphere of pure oxygen. Vac = vacuum.

Table I—*continued*.

Fig. 1, curve No.	Experi- ment No.	p_H .	Tem- perature.	Oxygen pressure, mm. Hg.*	Log I_0/I .		Per cent. oxy- chlorocruorin.
					$\lambda=604.5 \mu\mu$.	$\lambda'=580.5 \mu\mu$.	
5	24	7.7	10	Vac.	0.587	0.635	0
				Ox.	1.284	0.440	100
				25	1.236	0.479	88
				21	1.118	0.491	75
				18	0.999	0.522	58.5
				14	0.810	0.593	28.5
5	25	7.7	10	Vac.	0.590	0.629	0
				Ox.	1.242	0.441	100
				25	1.136	0.477	82.5
				16	0.845	0.575	36
				20	1.010	0.545	56.5
				14	0.792	0.595	27.5
6	7	7.7	20	21	1.028	0.524	63.5
				Ox.	0.432	0.133	100
				Vac.	0.220	0.219	0
				10	0.250	0.220	9
				12	0.256	0.215	11
				27	0.340	0.177	53
6	8	7.7	20	Ox.	0.440	0.140	100
				Vac.	0.240	0.253	0
				38	0.429	0.167	79.5
				32	0.440	0.191	74.5
				25	0.350	0.221	43
				18	0.290	0.210	31
6	10	7.7	20	Vac.	0.442	0.486	0
				Ox.	0.945	0.353	100
				26	0.677	0.401	52
6	13	7.7	20	Ox.	1.050	0.471	100
				Vac.	0.625	0.661	0
				41	1.060	0.520	89
				41	1.030	0.496	91.5
				32	0.935	0.544	68
				26	0.897	0.620	47.5
7	17	7.35	26	Ox.	1.049	0.412	100
				42	0.943	0.417	86
				36	0.895	0.439	74
				20	0.697	0.522	26
				Vac.	0.635	0.608	0
7	18	7.35	26	Ox.	1.430	0.485	100
				Vac.	0.750	0.804	0
				47	1.422	0.530	92
				33	1.257	0.604	63
				28	1.124	0.689	47
7	22	7.35	26	Ox.	1.239	0.442	100
				Vac.	0.640	0.654	0
				30	1.003	0.545	57
				45	1.203	0.487	86.5
				29	0.950	0.583	45
				21	0.785	0.623	21

* Ox = atmosphere of pure oxygen. Vac = vacuum.

The limiting temperatures studied, 10° and 26° , were chosen because the former is the winter sea temperature in Brittany, the latter the August sea temperature at Algiers (personal communication from Dr. Maurice Rose). These are extreme temperatures in the habitat of *Spirographis spallanzanii*. The p_H extremes of the experiments were dictated by the fact that chlorocruorin is unstable above p_H 8.5 and below p_H 7.0. For the reason given above it was necessary to study diluted blood, but owing to the dilution it is probable that the oxygen affinities obtained have lower values than those for chloro-

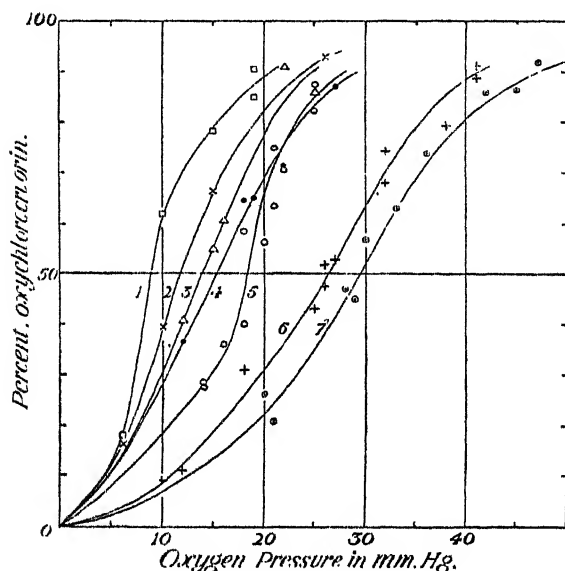


FIG. 1.—Oxygen dissociation curves of chlorocruorin. 1, p_H 8.0, 10° ; 2, p_H 8.2, 20° ; 3, p_H 8.0, 20° ; 4, p_H 8.0, 26° ; 5, p_H 7.7, 10° ; 6, p_H 7.7, 20° ; 7, p_H 7.35, 26° .

cruorin in whole blood. Ox hæmoglobin, for example, is 50 per cent. saturated with oxygen at an oxygen pressure of 5 mm. Hg in blood diluted to 1 in 31 with borate buffer solution, but it is 50 per cent. saturated at 7.5 mm. in whole blood, at similar p_H and temperature (Forbes and Roughton, 1931).

It is apparent from fig. 1 that the oxygen dissociation curves of chlorocruorin are sigmoid and are similar to those of hæmoglobin. The oxygen affinity of chlorocruorin at the temperature at which the animal lives is of the same order of magnitude as that of hæmoglobin in the human body, for human whole blood at 38° is 50 per cent. saturated with oxygen at an oxygen pressure of 29 mm., in presence of 40 mm. of CO_2 (Brown and Hill, 1923), and the same is true for diluted *Spirographis* blood at 26° and p_H 7.35. This similarity could not have been foreseen, since chlorocruorin is not fully saturated with oxygen

when equilibrated with air at atmospheric pressure. By comparing the total oxygen capacity of *Spirographis* whole blood equilibrated with air with that of blood equilibrated with oxygen, I had previously found that chlorocruorin is only 90 per cent. saturated in air (Fox, 1926). The average result of six experiments with diluted blood, using the present spectrophotometric technique, gave 96 as the percentage of oxychlorocruorin in the solution equilibrated with air. The difference between the two figures may be due to experimental error, but the higher figure in the present investigation may also be due to the fact that the blood was diluted, and would therefore, as explained above, be expected to have a greater oxygen affinity than whole blood. But, at all events, a respiratory pigment not fully saturated with oxygen in air might have given dissociation curves quite unlike those found for chlorocruorin. The hæmocyanin of *Loligo peali*, for example, in presence of 6 mm. CO_2 at 22° , is only 90 per cent. saturated with oxygen in air, and it is 50 per cent. saturated at as high an oxygen pressure as 97 mm. (Redfield, *et alii*, 1926).

Fig. 1 shows that both temperature and hydrogen ion concentration affect the oxygen affinity of chlorocruorin in a similar manner to mammalian hæmoglobin. The oxygen affinity is lessened by a rise in temperature and by a decrease of p_{H} . Between 10° and 20° the temperature coefficients (Q_{10}) of the reaction between chlorocruorin and oxygen (in the buffer solutions used), calculated from the oxygen pressures corresponding to 50 per cent. saturation, are 1.6 at p_{H} 8.0 and 1.5 at p_{H} 7.7.

While the p_{H} effect on oxygen affinity is the same for chlorocruorin and for mammalian hæmoglobin, this is not so for all hæmoglobins. The oxygen affinity of the hæmoglobin of the echiuroid worm *Urechis caupo*, an animal more nearly related to *Spirographis* than are vertebrates, is unaffected by CO_2 (Redfield and Florkin, 1931).

Curves 1 and 7 in fig. 1 represent the approximate limits of the oxygen affinity of chlorocruorin in the living *Spirographis*. The distribution of animals possessing and requiring respiratory pigments must be limited in part by the properties of these substances. Fishes living in situations where the oxygen supply is often poor have bloods with a high affinity for oxygen (Krogh and Leitch, 1919; Root, 1931). The same is the case for *Arenicola* (Barcroft and Barcroft, 1924), for *Chironomus* (Leitch, 1916) and for *Limulus* (Redfield, *et alii*, 1926). Animals which, on the contrary, have respiratory pigments with relatively low oxygen affinities may be more active because the oxygen is more readily given up to the tissues, but they cannot live in situations deficient in oxygen. Such are the trout (Krogh and Leitch, 1919) and the squid (Redfield,

et alii, 1926). Moreover, the effect of temperature on the dissociation curve may be a limiting factor in geographical distribution. The rate of diffusion of oxygen from blood to tissues depends on the difference in oxygen pressure between the two. It may well be that *Spirographis* could not live in seas having a temperature higher than about 26° because the oxygen dissociation curve would then be shifted too far to the right, and consequently the oxygen pressure difference between blood and tissues would be insufficient for life. From this point of view it is proposed to study oxygen dissociation curves in relation to temperature in closely related species of animals limited in their distribution to different latitudes.

The results of the investigation described above are not only a further step in the comparison of chlorocruorin with hæmoglobin, but they are an essential preliminary to an investigation now in progress of the functions of chlorocruorin in the life of animals possessing this pigment.

This investigation was done in the laboratory of Dr. René Wurmser in the Institut de Biologie Physicochimique in Paris. I wish to express my warmest thanks to Dr. Wurmser, to Mr. Louis Rapkine and to Mlle. Nélícia Mayer for continual help given to me in the course of my work.

Summary.

Using a spectrophotometric technique, the oxygen affinity of *Spirographis* chlorocruorin has been studied within the limits of temperature and p_H probable in the life of the animal.

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The Climbing Organ of an Insect, Rhodnius prolixus (Hemiptera : Reduviidæ).

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Adhesive or climbing organs are familiar structures in many groups of insects. Most commonly, as in Hymenoptera, Diptera and many Hemiptera, they take the form of empodia or pulvilli between the tarsal claws ; in a few Hemiptera they occur at the lower end of the tibia (Weber, 1930), while in many Coleoptera and Orthoptera it is the ventral surface of the tarsal segments themselves which is specially modified (Dewitz, 1884).

These structures are generally stated to be absent in the Reduviidæ, but one of us (Gillett, 1932) has recently observed a new type of climbing organ in the blood-sucking reduviid bug, *Rhodnius prolixus* Stål. The object of the present paper is to describe the structure of this organ and to discuss its mode of action.

Attention was first drawn to the organ in question as it was noticed that only the adult *Rhodnius* could climb the glass walls of their jars, while the nymphs at all stages were quite unable to do so. It was soon discovered that this ability to climb smooth surfaces was due to the existence in the adult insects of a small "fleshy pad" situated at the lower end of the tibia of the first two pairs of legs. This pad is absent from the third pair of legs in the adult, and is entirely wanting in the nymphs.

The organ is mentioned by Stål (1859), in his original description of *Rhodnius*, as a depression ("tibiæ anteriores apice subtus fossula spongiosa parva instructæ"), but in the living insect it is an elastic sac distended with fluid. It is of a dull orange colour, oval in outline, about 400 μ long by 300 μ broad (the adult *Rhodnius* measures 2 cm.). Its relation to the tibia and tarsus is shown in fig. 7.

Stål makes no suggestion as to the function of this organ, and del Ponte (1920) adds nothing to Stål's description. Pinto (1925), in his monograph on the blood-sucking Reduviidæ, does not mention it. An homologous structure, known as the "fossa spongiosa tibiæ," is present in the majority of Reduviidæ, but there seems to be no information available as to its function.

The Function of the Climbing Organ.

When an adult *Rhodnius* ascends a steep glass slope it uses the anterior and middle pairs of legs to draw itself upwards ; the hind legs either make unavailing attempts to grip the glass or just hang motionless. There is no doubt, therefore, that it uses the tibial pad for climbing. But in order to gain some idea of the efficiency of the organ, we performed the following experiments :—

A glass tube 1 inch by 5 inches was thoroughly cleaned inside, and a first-stage nymph placed in it.* As the insect crawled along, the tube was slowly raised at the end to which it was crawling. It immediately attempted to run, its feet slipping on the shiny surface, until there came a point at which it was unable to make any further progress. At this stage the angle between the tube and the surface of the bench was measured. This angle differed slightly for each individual, but never exceeded 10°. The experiment was repeated, using larger nymphs, and similar results were obtained, Table I.

Table I.

Stage of insect.	Number tested.	Maximum angle.	Minimum angle.	Average angle.
First-stage nymphs	15	8	6	6 30
Middle stages of nymphs	15	10	8	9 20
Last-stage nymphs	15	10	5	6 50
Adults	15	89	86	88 0

All the nymphs attempted to run up the shiny slope ; but the behaviour of the adults was strikingly different. Not one of them attempted to run, but each one slowly and steadily climbed the tube at almost a right angle, Table I. When placed at a right angle, they did not fall off, but very slowly slipped down. If the tube was jerked while they were climbing, instead of falling to the bottom, like the nymphs, they slipped a little way, and then suddenly stopped and resumed their climbing.

The adult *Rhodnius* cannot hold to the lower surface of clean glass, but it can hold and walk along the lower surface of cork or paper, as can also the nymphs, but if the insect is viewed horizontally it can be seen to be gripping with the claws, while the tibial pad is no longer in contact with the surface. The pad is used, therefore, only on smooth surfaces.

* The first-stage nymphs are 2.5 mm. long and weigh 0.5 mg. ; the last-stage nymphs are 1.3–1.5 cm. long and weigh about 50 mg. ; the adults are 2 cm. long and weigh about 90 mg.

In view of the possible mechanisms involved, which will be discussed later, it was of interest to discover whether the organ is equally efficient when the insect is climbing *down* a polished slope. The experiments were performed in the same tubes as before. They were a little difficult to carry out because on raising the glass tube at the end from which the insect was turned, it always swung round on its front legs and began to crawl upwards. This difficulty was overcome by leaving the insect in the tube all night at room temperature (they were normally kept at 24° C.), and next morning it was sufficiently inactive for one end of the tube to be raised without disturbing it. Table II shows the angle required to *start* it slipping forwards: this, of course, is greater than that which would be required to keep it moving when once it had started. Occasionally, one would slip straight down without swinging round, and then the tube could be lowered until the bug was only just moving. This happened at an angle of about 22°.

Table II.

Number of insects tested.	Maximum angle.	Minimum angle.	Average angle.
10	° 38	° 34	° 36

From these results it is evident that the organ is of little use to the insect when climbing downwards on a smooth surface.

It has been suggested that the climbing organs of many insects secrete a fluid which plays an essential part in their mechanism. Such a fluid might have the wetting properties of water, or of oil, or it might be a sticky or cohesive substance. Neglecting the third possibility for the moment, some evidence as to whether the hypothetical fluid is oily or watery may be gained by testing the ability of the insect to climb a waxed surface; for a watery fluid should not adhere to wax, and if a watery fluid is an essential part of the mechanism, the insect should fail to climb such a surface.

The experiments were carried out as before but with the tubes lined with a thin coating of paraffin wax. Under these conditions the adults could climb at an angle varying in different individuals from 70°–90°. When climbing near their maximum angle, their legs slowly slipped downwards, but the speed at which they were travelling upward exceeded the downward movement, so that they slowly rose. The nymphs managed to climb an angle of about 55°; except in the first stage when they could climb a vertical surface, Table III.

Table III.

Stage of insect.	Number tested.	Maximum angle.	Minimum angle.	Average angle.
		°	°	°
First-stage nymphs	15	90	90	90
Middle stages of nymphs	15	59	53	55
Last-stage nymphs	15	73	43	56
Adults	15	90	70	77

These results are necessarily confused by the fact that the surface is not entirely smooth, so that the insects could make use of their claws, which helped especially the very light first-stage nymphs. They do, however, show clearly that the climbing organ can function on a waxy surface, and therefore they suggest, as far as they go, that any secretion produced must be of such a nature that it will adhere to wax, that is, either an oily secretion, or a watery secretion containing some substance which causes it to spread on wax.

The experiments so far described have shown that the climbing organ is functional only if drawn backwards, as when the insect is ascending a slope. It offers comparatively little resistance to being drawn forwards, as when the insect is descending a slope, and it does not hold to the surface if this is displaced vertically, for the insect cannot cling to the lower surface of clean glass. It is possible that an oily secretion is concerned in these properties.

These same conclusions have been reached in another way. If the insect is held down on its back by a band of plasticene across the thorax, and the front legs held extended by further strips of plasticene, the climbing organs can be studied under the binocular dissecting microscope. A clean fragment of a coverslip may be mounted in a small piece of plasticene at the end of a dissecting needle and pressed down upon the tibial pad. Then it is found that the glass does not adhere to the organ in the slightest degree, showing the absence of any sticky secretion. Nor is there any appreciable resistance if it is moved backwards over the surface of the pad, that is, away from the tarsus. On the other hand there is definitely more resistance, as may be judged from the fact that the fragment of glass may be detached from its holder, if it is moved forwards, that is, towards the tarsus.

Further, on examining the glass after this procedure, it is found to bear a greasy smear. This dissolves instantly in xylol, leaving an oily residue on evaporation. It dissolves with difficulty in alcohol, and is quite insoluble in water, floating as a film on the surface. It is of some importance to note that

the smear is much more distinct when the glass is moved backwards over the pad, whereas when the glass is moved forwards scarcely a trace of grease may be seen. This, no doubt, accounts for the fact that the insect does not normally leave footprints when it walks on glass. The explanation of this difference will be apparent later.

The foregoing experiments leave no doubt that the tibial organ of *Rhodnius* is used in climbing. But, as already noted, it is wanting in the nymphs, and this raises the question whether it subserves any other purpose. In the adult *Rhodnius* it is present and equally developed in both sexes, but Stål (1859) observed that a similar structure in certain other Reduviid bugs is confined to the male; this suggests that it may serve also, or perhaps primarily, as a sexual clasping organ, comparable with that on the tibia of the male *Dytiscus*.

During copulation the male *Rhodnius* approaches the female from the side. His hind legs, which are devoid of the climbing organ, lie flat across the wings and abdominal sternites of the female; the middle and fore pairs of legs do not touch the female except at the climbing organ and at the claws. It is probable, therefore, that the organ may be of service to the male in holding the female, but it is not so used by the female herself.

A few observations have been made, for comparison, on the closely allied insects, *Triatoma rubrofasciata* and *T. flavida*.* In *T. rubrofasciata* the organ is similar to that of *Rhodnius* in the male, but is entirely wanting in the female. On clean glass the male can climb at an angle of about 18°, the female at an angle of about 8°. In *T. flavida* the organ is well developed in the male, but very much reduced in the female. The male *T. flavida* can climb clean glass at an angle of about 60°, and the female at about 5°. Though not so efficient in these species as in *Rhodnius*, the structure clearly functions as a climbing organ also in *Triatoma*.

The Structure of the Climbing Organ.

The structure of the climbing organ has been studied by dissection, by mounting the whole organ in Canada balsam, euparal and de Faure's medium, and by cutting transverse, longitudinal and coronal sections. These were fixed in Carnoy and cut in paraffin, and stained with Ehrlich's hæmatoxylin and eosin. The best results were obtained from insects which had recently moulted. The following account describes the organ as it occurs on the first pair of legs, but that on the middle pair has substantially the same structure.

* *T. rubrofasciata* is 1.8 cm. long and weighs about 90 mg.; *T. flavida* is about 2.6 cm. long and weighs about 230 mg.

Fig. 1 shows the organ as seen in longitudinal section. It has a sole plate of very thick but pliant chitin in which are set a great number of cylindrical spines or tubes. By counting the number of these in a measured area of a coronal section and relating this to the whole area, we have estimated that there are between 5000 and 6000 of these spines in each organ. At the anterior end of the pad they are long and curve backwards, while further back they become progressively shorter and straighter.

The minute structure of the spines is shown in figs. 2, 3 and 4. They do not arise from sockets like ordinary bristles, but each runs stiffly through the chitinous sole plate like the bristles set in a hair brush. Each spine is about $1\ \mu$ in diameter, uniform throughout its length save at the tip where it becomes somewhat dilated and has the anterior surface cut away obliquely. They appear to be tubes, open at the end, but they are so small that this point cannot be decided with any certainty by optical means.

In addition to these curved cylindrical spines with oblique ends, there are a few straight spines, fig. 5, which taper gradually towards their free extremity and project very slightly beyond the others. These show no indication of a terminal opening. They become somewhat stouter at the base and arise from large conspicuous sockets. From whole mounts and serial sections we have estimated their number to be about 50 or 60. They are distributed irregularly over the pad, usually separated by eight or nine of the rigid spines, but sometimes almost contiguous to one another.

The sole plate is about $10\ \mu$ thick, and made up of a great number of lamellæ. Its outer part usually stains with eosin, its inner part with hæmatoxylin. In horizontal sections through this plate the rigid spines appear as pores.

Above the sole plate is a conspicuous glandular epithelium, which comprises two distinct elements, connected respectively with the two types of spines, fig. 6. Over the rigid spines there are densely packed cells with little cytoplasm and relatively large nuclei. It is possible to make out ducts running from the bases of the spines into these cells; but the cells are so congested that we have been unable to determine their precise relation to the ducts. To the best of our judgment each duct is connected with a single cell.

Above the large sockets of the straight spines there is a pear-shaped structure which is presumably a sense organ. This consists of a small vesicle above the socket, surmounted by a spindle-shaped mass of cells from the apex of which a nerve fibre is given off. A well defined filament, which usually stains with eosin, can be traced from the base of the spine, through the socket and into the vesicle. Here it runs a more or less convoluted course, and ends in the spindle-shaped mass of cells.

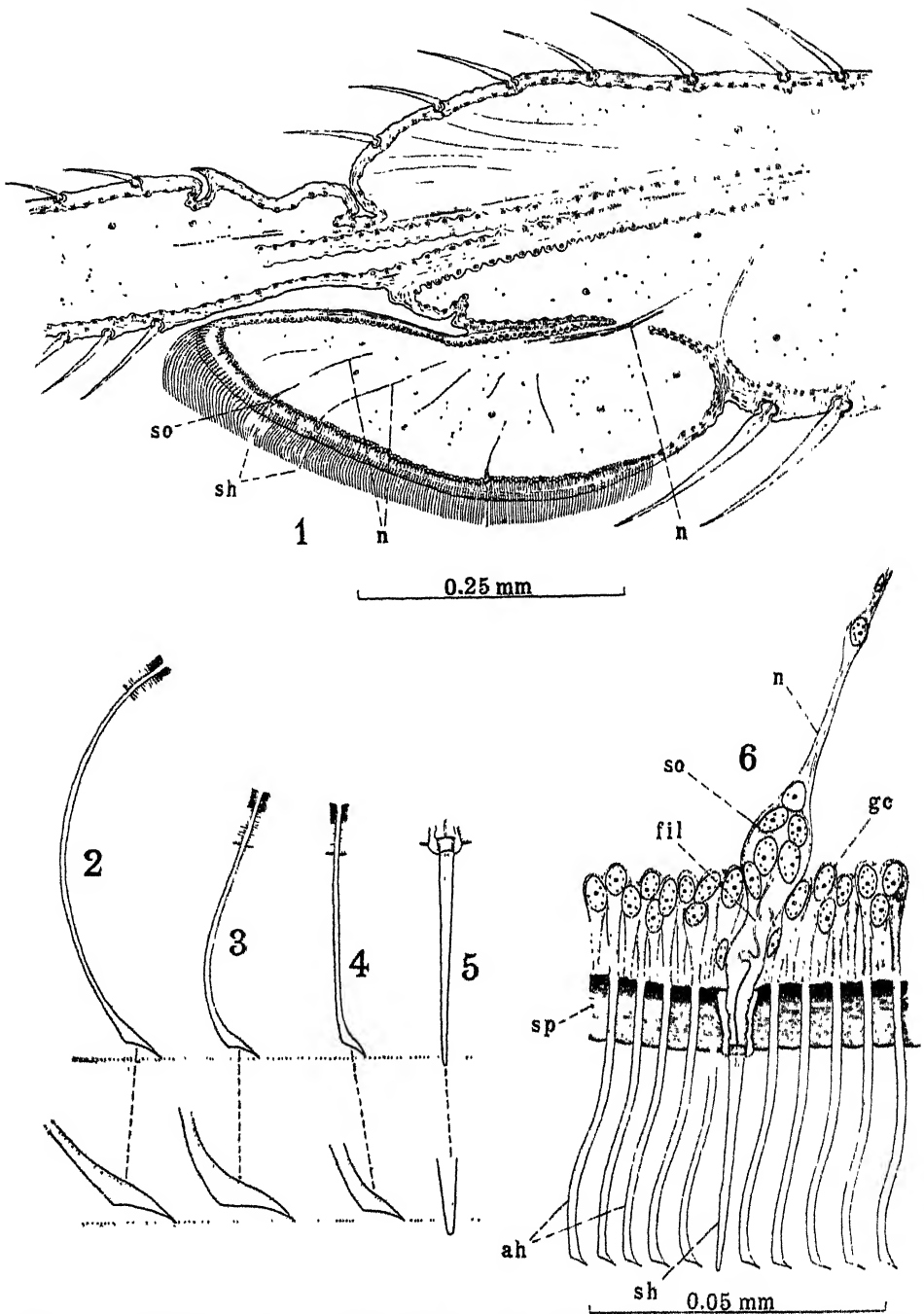


FIG. 1.—Longitudinal section of climbing organ. FIGS. 2, 3 and 4.—Single adhesive hairs from different parts of climbing organ, with detail of terminal portion of each. FIG. 5.—Sensory hair. FIG. 6.—Longitudinal section of climbing organ to show detailed structure. *ah*, adhesive hairs; *fil*, coiled filament from base of sensory hair; *gc*, glandular cells; *n*, nerve fibres; *sh*, sensory hair; *so*, sense organ; *sp*, chitinous sole plate of climbing organ.

There can be little doubt that these structures are sense organs. In general appearance they are like the "tactile hairs" mentioned very briefly by Dewitz (1884) in the tarsal climbing organ of *Telephorus*, Col. The only difficulty about this interpretation is that they are far more complicated than tactile hairs in general, and approximate much more closely to the type of sense organ which Berlese (1909) regards as olfactory. But until the precise function of the different types of insect sense organs is better understood, too much emphasis cannot be put upon such morphological distinctions.

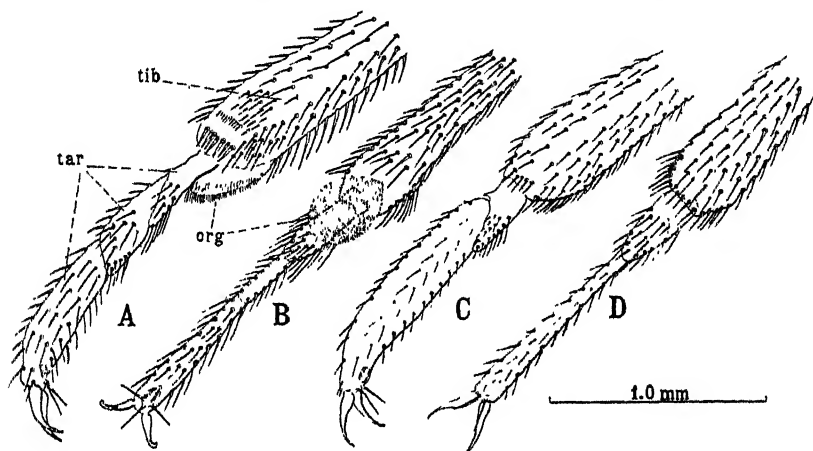


FIG. 7.—A, lateral view of lower end of an anterior tibia and tarsus of adult *Rhodnius* ; B, ventral view of same ; C, lateral view of anterior tibia and tarsus of last stage nymph ; D, ventral view of same. *Org.*, climbing organ ; *tar*, tarsus ; *tib*, tibia.

The cavity of the climbing organ is filled with blood, and is continuous with the cavity of the tibia through a pore at its upper surface. The pad is, in fact, an evagination from the end of the tibia. The greater part of the roof is closed by a membrane. The nerve fibres from the sense organs run backwards below this membrane and eventually reach the tibia through the pore already mentioned. No tracheæ or muscle fibres could be seen to enter the climbing organ.

These histological observations suggest that the rigid cylindrical spines with oblique ends are tubes which discharge the secretion of unicellular glands within the climbing organ ; and that among these there are a few sensory bristles which may enable the insect to appreciate the nature, and possibly the inclination, of the surface upon which it is walking.

The Mechanism of the Climbing Organ.

The mechanism of the climbing or adhesive organs of insects was extensively studied about fifty years ago, but very little seems to have been added in

more recent times. Full historical accounts of the earlier work are given by Tuffen West (1862) and Dahl (1884). The ideas advanced by the later observers may be summarised as follows: Dewitz (1884) held that in every case the adhesive organs secrete a sticky fluid, and that it is the *cohesion* of this fluid which holds the insect to the surface. Dahl (1885) believed that the organs allow "altered blood" to percolate through invisible pores on their surface, and that the extremities of the hairs or tubes of the adhesive organ, being very soft and delicate, can be pressed against the surface so closely that in the presence of a very small amount of fluid they adhere—that is, the insect is supported by *adhesion* between the hairs and the surface. Tuffen West (1862) and Simmermacher (1884) considered that the organs always function as suckers, *i.e.*, that they are held to the surface by the *atmospheric pressure*, and that although in some cases a fluid is present, this serves merely to make the junction between the tubes and the surface airtight. Rombouts (1884) believed the force concerned to be the *surface tension* of the fluid secretion. This he supposed to act around the margins of the individual hairs, holding them to any surface that could be wetted by the secretion in question.

A general discussion of these mechanisms is not called for here, for it is not improbable that the mechanism varies in different insects. But the climbing organ of *Rhodnius* has properties which enable us to obtain more certain information as to its mode of action. Diagrammatically, it consists of a number of obliquely truncated cylinders provided with an oily secretion, fig. 8, A. These cylinders will slide easily over the surface of glass in the direction

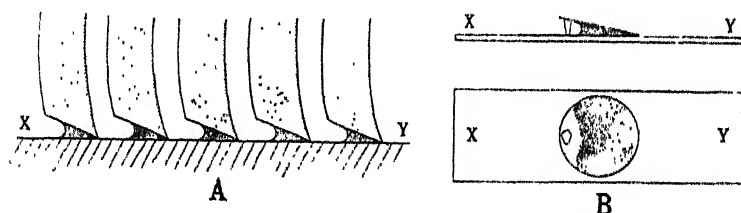


FIG. 8.

X, as when the insect is inclined head forwards on a *downward* slope; but they offer great resistance to sliding in the direction Y, as when the insect is inclined head forwards on an *upward* slope. The organ functions well on glass, less well on paraffin wax.

The first explanation that suggests itself is that of a ratchet: the oblique points of the hairs sliding over any irregularities in the surface when moved towards X, but catching in these when moved towards Y; this idea is, however,

not applicable in the case of smooth glass, which has no asperities of sufficient magnitude.

It is more likely that surface forces of some kind are at work, and the question turns on whether a movable disc separated from a fixed plate by a wedge of oil will offer greater resistance to movement towards the point of the wedge, *i.e.*, towards Y, than in the reverse direction, towards X.

Now according to the theory of "film lubrication" developed by Osborne Reynolds (1883), the optimal conditions for lubrication obtain in a bearing when the bearing surfaces are separated by a wedge-shaped space through which the oil passes from the wide inlet to the narrow outlet, an arrangement that finds practical application in the Michell Thrust Bearing as used at the present day in hydro-electric turbines (Boswall, 1928). Thus it follows that when the inclined disc that we are considering is moved towards X, the conditions are ideal for lubrication and the frictional resistance will be at a minimum.

When the inclined disc is moved towards Y, the converse holds. The layer of oil in immediate contact with the fixed surface will adhere to this and will be held back, bringing the advancing margin of the disc closer to the fixed surface. This will happen to successive layers of oil, and consequently the oil film at the apex of the wedge will become thinner and thinner until, finally, it will break and seizure will occur.

These effects can be demonstrated very clearly by means of a model, fig. 8, B, consisting of a circular coverslip resting on a glass plate. The coverslip is raised at one side with a small fragment of glass cemented to its lower surface, and a drop of olive oil coloured with Sudan III is run underneath it. It is then found that the coverslip moves easily in the direction X, but offers great resistance to movement in the direction Y. This resistance does not develop until the coverslip has been moved a few millimetres towards Y, and as it develops the layer of oil at the advancing margin of the coverslip becomes thinner (as shown by the disappearance of colour); finally, when the resistance reaches its maximum, Newton's rings appear around the advancing point. The advancing point itself usually lies in a black spot at the centre of the rings, showing that here the surfaces are in exceedingly close contact. This strongly suggests that seizure is occurring in this area between the coverslip and the glass plate.

The experiment may be made very striking by cementing a 10-gram weight on to the coverslip and connecting it, by means of a thread passing over a smooth glass rod, with a scale pan, fig. 9. In one such experiment it was found that with the apparatus arranged as in fig. 9, A, *i.e.*, with the advancing margin

of the coverslip raised, it would just support a weight of 1 gram in the scale pan ; but, at the slightest touch, equilibrium was destroyed and the weighted coverslip was drawn rapidly to the top of the slope. Whereas, with the apparatus arranged as in fig. 9, B, with the advancing margin of the coverslip

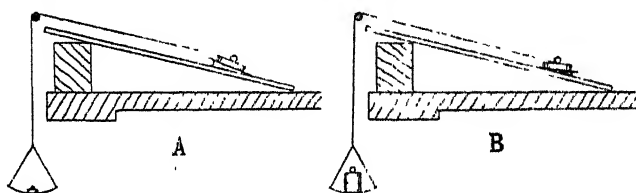


FIG. 9.

lowered, it would support 40 gm. in the scale pan and the weighted coverslip crept almost imperceptibly up the slope.

Further, if a weighted coverslip prepared as above is placed on a sheet of glass, which is gradually inclined, then when the *lower* margin of the coverslip is raised, fig. 10, A, it will not hold at an angle greater than about 5° ; whereas when the *upper* margin is raised, fig. 10, B, the glass plate can be inclined beyond a right angle and the coverslip does not fall off, although it usually creeps very slowly downwards.

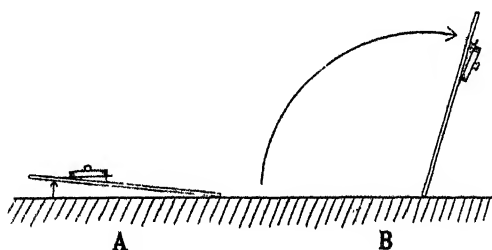


FIG. 10.

The model acts in the same manner on a smooth block of paraffin wax. Whether it offers greater or less resistance on this than on glass cannot be determined because the edge of the coverslip cuts into the surface of the wax and increases the resistance in this way.*

All the properties of this model are so similar to those already described in the living *Rhodnius* as to suggest very strongly that the same principle is

* It is interesting to note that Dr. N. K. Adam has found (in unpublished work to which we are kindly permitted to refer) that *W* (the "work of adhesion") is greater for oil on glass than for oil on wax. It is possibly for this reason that *Rhodnius* is less efficient at climbing on wax than on glass (see Tables I and III).

involved in both. Thus, if the explanation given in the case of the coverslip is correct, *i.e.*, that the force resisting movement is seizure or adhesion between the moving surfaces, then the mechanism of the climbing organ, also, depends probably on the adhesion of its minute elements to the surface upon which it climbs.

This mechanism is particularly well suited to the needs of the insect, for it is well known that by altering slightly the direction of the force, it is easy to separate two surfaces that have seized (see Adam, 1930, p. 215). The insect will therefore have no difficulty in detaching the climbing organ as it walks.

Summary.

The climbing organ of *Rhodnius prolixus* is present in both sexes of the adult but is absent in the nymphs. It occurs on the distal end of the tibia of the anterior and middle pairs of legs. It enables the insect to climb upwards on clean glass at almost a right angle, but it is of little use in the reverse direction, *i.e.*, when the insect is going down a slope.

The organ is a little oval sac of pliant chitin filled with blood. On its lower surface it bears about 5000 tubular hairs 1 μ in diameter, which appear to be the outlets of unicellular glands producing an oily secretion. At their free ends the anterior surface of these hairs is cut away obliquely so that only their hind margin comes in contact with the surface as the insect climbs.

Among these hairs are about 50 delicate tapering hairs arising from large sockets and projecting slightly beyond the others. These appear to be sense organs. They are surmounted by a spindle-shaped mass of cells giving off a nerve fibre.

To elucidate the mechanism of the climbing organ, a model has been constructed consisting of a disc separated from a glass plate by a wedge of oil. This can be moved readily in one direction (towards the open end of the wedge), but is very resistant to movement towards the point of the wedge. Evidence is brought forward that this is due to adhesion or seizure caused by the breaking down of the oil film at the point of the wedge.

It is suggested that the mechanism of the climbing organ of *Rhodnius* is the same as in this model.

We are much indebted to Dr. N. K. Adam for suggesting to us that "seizure" might be the explanation of the coverslip experiments, and for directing our attention to the relevant literature.

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The Effect of Atmospheric Humidity on the Metabolism of the Fasting Mealworm (Tenebrio molitor L., Coleoptera).

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INTRODUCTION.

Previous work has shown that fasting mealworms will live at room temperature for two hundred days, and even at 30° C. they usually live for over a month. During the first two days of starvation the mealworms are restless, and they pass a certain amount of excrement. After this they lie quite still, and pass extremely little excreta. The loss in weight of starving mealworms is different in dry and moist air at one temperature, or in air with the same relative humidity at two temperatures. At 23° C. the mealworms evidently regulate their metabolism, because while they lose weight at different rates in air of various relative humidities, yet they keep the ratio of dry matter to water in their bodies constant (Buxton, 1930).

In carrying this work further, I have attempted to find whether the rate at which fasting mealworms evaporate water is proportional at any temperature to the saturation deficiency of the air. Now the fasting mealworm not only evaporates water present in its body at the start of the experiment, but also considerable quantities of water produced by the metabolism of food reserves during starvation. We can estimate the amount of water present in the mealworms at the start of starvation, and can find how much is left at the end of the experiment; the difference represents *part* of the total water evaporated. But this method does not indicate any metabolic water which is produced and also evaporated during starvation. If one wishes to know the total water evaporated, it can be collected in a stream of air, or else the loss of food reserves must be estimated and the metabolic water produced calculated from these results. I preferred to use the second method, as it is not easy to measure the actual amount of water given off by insects except into dry air.

METHODS.

Hein's technique (1920) was used in breeding the mealworms, and they were fed on bran, dog-biscuit and turnip. The last caused them to grow more rapidly, but when it was withheld their body composition was less irregular; in consequence they were not given turnip for a week or more before the beginning of each experiment. The main disadvantage of mealworms for this kind of work is that their body composition is so irregular, and it was therefore necessary to use very large batches for analyses. It is important that analyses should give accurate average figures, since the actual mealworms which are starved cannot themselves be analysed till afterwards, and their initial composition has to be assumed from the analyses of others from the same culture.

The mealworms were kept separately in 3×1 -inch glass tubes during starvation, since it was found that, if kept in batches, some individuals died or pupated, and others bit one another. Any mealworms which died or pupated during the experiments were discarded. I used mealworms of all sizes from 19 mg. up to 274 mg., but each batch used consisted of mealworms of nearly the same size taken from the same culture. Other mealworms from each culture were analysed at the same time that a batch was starved. The larger the mealworm, the greater the accuracy in weighing and in analysis; but larger ones have a greater tendency to pupate. There seems, however, to be no hard-and-fast rule as to what size a mealworm should reach before it pupates. Some

mealworms of over 200 mg. did not pupate during several weeks of starvation, while others of under 100 mg. did so.

The tubes containing the mealworms were stood in glass desiccators, which were kept in thermostatically-controlled rooms. The humidity of the air in the desiccators was controlled by KOH solutions, made up to give definite humidities, as described by Paranjpe (1918). Buxton kept mealworms in the same way as I did, except that he controlled the humidity with sulphuric acid solutions; he suggested that the use of KOH would be an improvement, because it would absorb any CO_2 as it was produced. He felt that his results were open to criticism, because the CO_2 produced might open the spiracles and cause the mealworms to lose more water. I found, however, that at any particular temperature and humidity they lost the same weight (with KOH in the desiccators) as those in Buxton's experiments: his results, therefore, are evidently satisfactory.

LOSS IN WEIGHT AT CONTROLLED TEMPERATURES AND HUMIDITIES.

A summary of the results obtained by starving mealworms under controlled conditions is given in Table I. Some of the results duplicate those of Buxton (1930), and I have, where possible, included his results with mine, and it will be seen that they agree closely. He also kept mealworms at 23°C . and various humidities. I did not repeat these experiments, but have included his results in the discussion on the rate of loss of water (p. 387).

Table I.—Loss in Weight of Starved Mealworms.

—	Number of mealworms.	Days starved.	Relative humidity per cent.	Temperature, $^\circ\text{C}$.	Weight at end as percentage of original weight.	Buxton's figure for same conditions.
1	15	28	0	8	92.0 ± 0.6	—
2	12	28	30	8	93.0 ± 0.8	—
3	20	28	60	8	95.3 ± 0.23	—
4	16	28	90	8	112*	—
5	17	28	0	30	71.0 ± 0.70	70.6
6	58	26	30	30	75.3 ± 0.2	75.5
7	80	28	30	30	73.0 ± 0.15	74.0
8	85	26	60	30	82.0 ± 0.14	82.5
9	9	14	70	37	87.0 ± 0.49	—

Note.—6 and 7 were mealworms from different cultures.

* Gross increase in weight. A few individuals lost weight, the others gained.

CHEMICAL COMPOSITION.

Unstarved mealworms were analysed, and also others which had been starved at controlled temperatures and humidities. These analyses made it possible to show which of the body constituents were utilised during starvation.

1. *Normal.*

(a) *Dry Matter.*—The first thing I estimated was the proportion of dry matter. Buxton has shown that the proportion of dry matter in mealworms from the same culture varies widely. He dried 46 individuals of between 61 and 155 mg. at 100° C. over phosphorus pentoxide at reduced pressure, and found that the dry matter varied between 34 and 49·5 per cent., the mean proportion being $42\cdot8 \pm 0\cdot491$ per cent.

I dried the mealworms to constant weight in an oven at 105° C. This method gave consistent results, which agreed closely with Buxton's. In Table II is shown the proportion of dry matter found in various batches of mealworms which had been starved for only two days (except in the case of the newly hatched ones, which were dried within 24 hours of hatching). It appears that small mealworms have a lower proportion of dry matter than large ones, and this agrees with the findings of Teissier (1931). In the results in Table II marked with an asterisk, the mealworms were all from the same culture, (4) being small, (8) large, but (6) and (7) were not selected in any way. These results show that the larger ones had a greater proportion of dry matter; but it is always necessary to analyse any particular culture, for as (5) on the table shows, mealworms from a different culture from (6) and (7), but of very nearly the same average weight, have a much higher proportion of dry matter.

Table II.—The Proportion of Dry Matter in Normal Mealworms.

—	Number of larvæ.	Average size, mg.	Range of sizes, mg.	Percentage of dry matter.
1	56	0·55 (newly hatched)	—	24·2
2	44	1·4	—	36·2
3	40	58	19–102	37·6
4	66	88	60–100	40·51*
5	23	118	80–160	42·4
6	600	125	60–190	41·2*
7	500	127	60–190	41·3*
8	66	174	Over 150	42·2*

* From same culture.

(b) *Fat*.—The mealworms were dried at 105° C., ground up, and the fat, a yellow transparent oily liquid, extracted with ether in a Soxhlet apparatus. Dried mealworms from batch 6 in Table II contained 36 per cent. of fat, corresponding to 14.8 per cent. of their live weight; this result was found consistently in several analyses. Smaller mealworms, as batch 3, had a lower proportion of fat, 31.8 per cent. of their dry weight, and the very small mealworms, batch 2, had only 13 per cent.

(c) *Carbohydrate*.—I estimated the glycogen using Pflüger's method. The live mealworms were plunged into boiling 60 per cent. potash, which was then kept at 100° C. for several hours on the water bath, by which time all the body contents were in solution, and the exoskeleton was left quite clean. The mixture was filtered through glass wool, and the glycogen in the filtrate precipitated by the addition of alcohol. After hydrolysis with hydrochloric acid, the glycogen was estimated as glucose, using the method of Issekutz and Both's (1927). The glycogen usually amounted to about 2 per cent. of the live weight, or 5 per cent. of the dry weight. Figures of actual analyses are given in Table III.

Table III.—Carbohydrates Present in Mealworms.

(a) Glycogen.

	Percentage of glycogen—	
	Of live weight.	Of dry weight.
1. Living mealworms	2.04	4.9*
2. Dried mealworms	2.1*	5.0
3. Living mealworms	1.7	4.6*
4. Living mealworms .	1.57	4.15*

1 and 2 from the same culture.

(b) Sugars, etc.

	Percentage of sugars—	
	Of live weight.	Of dry weight.
1. Living mealworms ...	1.2	3.0*
2. Living mealworms	1.0	2.6*

* Calculated assuming dry weight obtained for that culture in Table I.

In addition to glycogen, there appear to be present some sugars or lower carbohydrates soluble in 80 per cent. alcohol. Some live mealworms were ground up with 80 per cent. alcohol, and the carbohydrates extracted and estimated after hydrolysis, the amount of reducing sugar found being equal to about 2.5 to 3 per cent. of the total dry weight of the animals. About half of this existed as a reducing substance, since it could be estimated without hydrolysing. The glycogen was precipitated by the 80 per cent. alcohol, and could be estimated in the residues. Results obtained in this way agreed very closely with those obtained by plunging the mealworms into the boiling potash, and the same percentage of glycogen was also found in mealworms that had been killed by drying in the oven at 105° C. But if mealworms are killed with the vapour of CS₂, the glycogen disappeared rapidly (in several hours), and erroneous results were obtained unless it was estimated at once.

(d) *Uric Acid*.—The easiest way to estimate the amount of protein used during starvation is by the products of its metabolism, since the nitrogen from digested protein is excreted in the form of uric acid, though some insects store considerable quantities of excretory products in their bodies. I therefore estimated the amount of uric acid present in the unstarved mealworms, and in others which had been starved. After starvation, any increase in the uric acid in the body and any uric acid in the small amounts of faeces passed showed how much protein had been consumed during starvation.

To extract the uric acid, the mealworms were dried and their fat removed. The uric acid was then extracted with water containing a very little sodium carbonate (Wigglesworth, 1925), and estimated colorimetrically by Benedict's method. There was very little uric acid present in the mealworms before starvation; it varied in different batches between 0.5 and 0.25 per cent.

2. *Starved.*

After the mealworms had been starved for periods of 26–28 days at 30° C., they were analysed for dry matter, fat, carbohydrates and uric acid, as described above.

The proportions of dry matter found are given in Table IV, and the amounts of fat, carbohydrate and uric acid in Table V. The latter table is made out in such a way as to facilitate the balancing of the loss of dry weight with the loss of its various constituents.

Table IV.—Dry Matter Present in Starved Mealworms.

Number of larvæ.	Length of experiment in days.	Relative humidity, per cent.	Temperature, °C.	Final weight as percentage of original weight.	Dry matter.	
					As percentage of final weight.	As percentage of original weight.
17	28	0	30	71	↗ 41.5*	↘ 29
58	26	30	30	75.3	39†	29.4
80	28	30	30	73	38.5‡	27
51	26	60	30	82	34	28
"small"						
34	26	60	30	82	37.5	30.7
"large"						
9	14	70	37	87	35.5	31

* Dry weight from 6 individuals.

† Dry weight from 9 individuals.

‡ Dry weight from 40 individuals.

There was a small amount of excrement passed during starvation, but the total weight was found to be very small, and after the first few days of starvation about 50 per cent. of it was in the form of uric acid.

I do not know precisely during which parts of the four weeks of starvation the various reserves are utilised. The glycogen, however, seems to be used mainly at the beginning, because batches analysed after a week's starvation were found to have reduced their glycogen content from 2.04 to 0.68 per cent. of their total weight. From the results given in Table V it is possible, however, to show which reserves are used up during starvation, and the balance sheet below, made up from the figures in the table, shows that we can account for over 90 per cent. of the reserves used.

	Gm:		Gm.
Original dry weight	2.671	By loss of fat	0.432
Final dry weight	1.990	By loss of carbohydrate....	0.138
		By loss of fæces and uric acid	0.054
Loss	= 0.681		0.624

It will thus be seen that fat was the principal reserve used, and this agrees with the work of Johansson (1920), who found that fasting mealworms had a respiratory quotient of 0.7.

Table V.—Analysis of Mealworms after Starvation for 28 Days at 30° C.

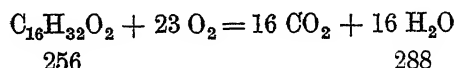
—	Tem- pera- ture, °C.	Relative humidity, per cent.	Gross weight.		Dry weight.		Fat.		Carbohydrates.				Uric acid.		Excreta.
			Beginning.	End.	Beginning.	End.	Beginning.	End.	Beginning.	End.	Beginning.	End.			
													Glycogen.	Sugar.	
1	30	60	Gm. 1.409 (17 individuals)	Gm. 1.1585	Gm. 0.486	Gm. 0.391	Gm. 0.175	Gm. 0.117	Gm. 0.025	Gm. 0.005	Gm. 0.016	Gm. 0.010	Gm. 0.0005	Gm. 0.0015	Gm. 0.008
2	30	60	Gm. 3.001 (17 individuals)	Gm. 2.4035	Gm. 1.260	Gm. 0.935	Gm. 0.454	Gm. 0.252	Gm. 0.060	Gm. 0.006	Gm. 0.038	Gm. 0.020	Gm. 0.00025	Gm. 0.010	Gm. 0.020
3	30	30	Gm. 2.467 (40 individuals)	Gm. 1.732	Gm. 0.925	Gm. 0.664	Gm. 0.295	Gm. 0.123	Gm. 0.044	Gm. 0.014	Gm. 0.028	Gm. 0.018	Gm. 0.0005	Gm. 0.0024	Gm. 0.012

Original dry weight, etc., calculated from unstarved mealworms of same culture. Carbohydrate and uric acid figures after starvation calculated from parallel batches.

METABOLISM OF FAT, CARBOHYDRATE AND PROTEIN.

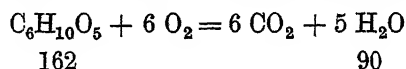
It has been shown which food reserves are used up during starvation, and the reactions below indicate how they are most probably metabolised in the mealworm.

Fat.—The composition of mealworm fat has not been determined, but probably it is not very different in empirical formula from palmitic acid (Uvarov, 1928). The reaction for the complete combustion of palmitic acid is as follows :—



which means that for every 6 parts of fat used, 7 parts of water of metabolism are produced in the body of the animal.

Glycogen.—The reaction for the combustion of glycogen is as follows :—



From this it will be seen that 162 grammes of glycogen yield only 90 grammes of metabolic water.

Protein.—Owing to the complex nature of proteins, it is not possible to give a satisfactory equation for their combustion, but, when used in an insect, protein yields uric acid, water and carbon dioxide. One gramme of protein will give half a gramme of uric acid and half a gramme of metabolic water ; therefore if the uric acid is excreted from the mealworm, each gramme is equivalent to a loss of 2 grammes in dry weight in the animal.

PRODUCTION OF METABOLIC WATER.

The amount of metabolic water produced depends upon the amount and nature of the food reserves used, the figures for which (see p. 382) are given below, with the amounts of water they produce on combustion.

Solid Matter Lost.	Water Produced.	
	Gm.	Gm.
Fat	0·432	0·504
Carbohydrate	0·138	0·077
Uric acid and fæces*	0·054	0·034
Total	0·624	0·615

* Fæces contained 50 per cent. uric acid (p. 382).

Thus when a mealworm is using its food reserves, if it does not evaporate any water its weight will remain practically constant. It is using fat, which alone would give an increase in weight, and carbohydrate, which alone would give a decrease, and the metabolic products of these two main reserves tend to balance each other, so that the decrease in weight of a starving mealworm is almost entirely due to evaporation. Several assumptions have been made regarding the constitution of the food reserves, but the result is not materially altered if different formulæ are substituted, and as more than half the water lost during the experiments was present in the mealworms at the beginning, any error in the calculation is halved. Thus, I consider that we are justified in assuming that loss in weight of a starving mealworm is almost entirely due to water evaporated, and no correction for the rate of metabolism is required.

RATE OF LOSS OF WATER:

Dalton's Law.

The rate of evaporation from a surface of water in still air follows Dalton's law, and it is possible that the rate of evaporation from a mealworm may be similarly determined. Dalton's law can be reduced to the statement that "under otherwise identical conditions, at all temperatures, loss by evaporation is directly proportional to saturation deficiency."

I have shown above that the loss in weight of a batch of mealworms during a period of starvation is very nearly the same as the weight of water which is evaporated from them. Thus, if the rate of loss of water is in accordance with Dalton's law, for any period the expression $\frac{\text{Loss in weight}}{\text{Saturation deficiency}}$ should be constant. In Table VI this expression has been calculated for the results given in Table I. The expression will be seen to vary only between 1 and 1.65, except in two cases, which are as follows: at 23° C. and 30 per cent. relative humidity, Buxton's figures give a result lower than most of the others, but that result was obtained with only five mealworms, and I consider that it may well be disregarded. The other result which does not agree is that which was obtained at 30° C. and 0 per cent. relative humidity. This was obtained by both Buxton and myself independently, with large numbers of mealworms. It will be noticed that at 23° C. and 0 per cent. relative humidity, these mealworms lost weight at the same rate as those at 30° C.; this may be the maximum rate at which water can be evaporated from a mealworm. The other results all agree roughly with one another. Thus it appears that the rate of loss of

water from mealworms at temperatures between 23° C. and 37° C., except in very moist or very dry air, follows Dalton's law.

I have not included in this table the results obtained at 90 per cent. relative humidity. Buxton, however, showed that at 90 per cent. relative humidity, at 30° C. and 23° C., starving mealworms increase in weight, which increase is sometimes large, amounting to as much as 18 per cent. of the insect's gross weight. The mealworms at 90 per cent. relative humidity and 8° C. also increased in weight. This increase was at one time considered to be caused by the insects producing more water of metabolism than they evaporated, but it is too great to allow of this explanation. Fat is the only food reserve which produces a greater weight of metabolic water than its own weight—1 gramme of fat yields $1\frac{1}{6}$ grammes of water. But the increase in weight of some mealworms at 90 per cent. relative humidity is much greater than the increase that could be explained by metabolism. It is evident that they must take up water from the air.

At low humidities the mealworms lose water at a rate proportional to the saturation deficiency of the air, while at high humidities—90 per cent. and over—they gain water. This phenomenon appears to depend on the relative humidity, and not on the saturation deficiency. Mealworms kept at 30° C. and 90 per cent. relative humidity increase in weight when the air has a saturation deficiency of 3.2 mm., but at 8° C. and 60 per cent. relative humidity the saturation deficiency is also 3.2 mm., and yet the mealworms all lose weight. Now inanimate objects which are hygroscopic take up definite weights of water depending on the relative humidity of the atmosphere, and absorb nearly the same weight of water at one relative humidity and any temperature (within certain limits). If such a substance has stood for a period at one relative humidity, so that it is in equilibrium with it, then, if it is removed to a lower relative humidity, it will give up water, while at a higher one it will absorb it. But the *rate* at which equilibrium is reached depends on the saturation deficiency, and so the higher the temperature (and for any particular relative humidity the higher the temperature the greater the saturation deficiency) the sooner the substance will reach a state of equilibrium. I believe that mealworms behave in a similar manner. I enclosed some in a small space with a weighing hygrometer (Buxton, 1931 *a*), and found that a batch came into equilibrium with the air at 88 per cent. relative humidity. Now I suggest that normal mealworms have a body constitution such that they behave like a hygroscopic substance that is in equilibrium with air of which the relative humidity is 88 per cent. Below 88 per cent. the mealworms lose

water, and the rate of loss is proportional to the saturation deficiency of the air, and above 88 per cent. they absorb water. The rate of uptake is, however, very irregular, and the water content of mealworms from any culture is also irregular; there may well be some relation between these facts. A mealworm is not completely like an inanimate object, because it does not reach equilibrium

Table VI.—Loss in Gross Weight of Batches of Mealworms at Various Temperatures and Humidities.

Temperature, °C.	Relative humidity, per cent.	Loss in weight as percentage of original weight.	Saturation deficiency in mm. of mercury.	Loss of weight. Saturation deficiency.
For 28 days.				
8	0	8	8	1.0
—	30	7	5.6	1.27
—	60	4.7	3.2	1.47
For 26 days.				
23	0	27	21	1.3*
—	20	24.4	16.8	1.45*
—	30	12.4	14.7	0.86*
—	60	10.5	8.4	1.25*
—	80	6.5	4.2	1.5*
30	0	27	32	0.84
—	30	24.7	21	1.2
—	60	18	13	1.4
—	80	10.5	6.3	1.65*
For 14 days.				
30	60	13	13	1.0
37	70	13	13	1.0

* After Buxton (1930).

with the air while it is alive. It is always producing water, either (at 23°) as fast as it is lost, or even faster (at higher temperatures). Thus, though it is tending to reach equilibrium with the air, its vital processes prevent it from doing so.

It is difficult to understand how an insect, the body fluid of which is in equilibrium with almost saturated air, can take up water from an atmosphere with a relative humidity of 90 per cent. It has been suggested that water collects in the tracheoles, and then asphyxiation causes the molecules in the body to split up and raise the osmotic pressure of the tissue fluids. But this cannot be the explanation, because mealworms kept at 30° C. and 90 per cent.

relative humidity use up the same amount of food reserves as do others at lower humidities ; their metabolic rates and their rate of respiration must be the same in all cases (see fig. 1). It is possible that the walls of the tracheal endings contain some hygroscopic substance. The walls are living membranes, and they might well work in one direction only. This would prevent undue evaporation in dry air, but in moist air, which is not a normal condition for the mealworm, water would be taken up. It is significant to note that current experiments on the bed-bug (*Cimex lectularius*) show that this insect, which inhabits damp cracks in walls for most of its life, *loses* water into nearly saturated air. If it were like the mealworm, it would be taking in water continually. The mealworm possesses some mechanism of a type not previously investigated, which is of value to it in its normal habitat, but it is not yet possible to postulate a definite theory as to how it works.

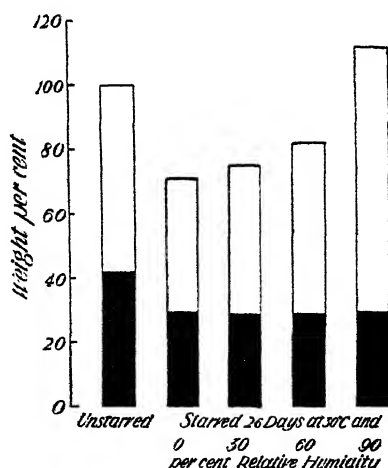


FIG. 1.—Mealworms before and after starvation for 26 days at 30° C. and several humidities. The black represents solid matter, and the white water.

During the first week of starvation the mealworms lose water more rapidly than subsequently. There are probably two causes of this. The metabolism is more rapid at the start, and though this in itself will not alter the weight, the spiracles will be opened more frequently, which will allow the water to evaporate at a greater rate. Also the glycogen is used up mainly during the first week, and as 162 grammes of glycogen only give 90 grammes of water on complete combustion, its use will cause a loss of weight irrespective of any evaporation of water. These two factors probably account for the more rapid fall in weight of the mealworms during the first week of starvation.

EFFECTS OF TEMPERATURE AND HUMIDITY ON THE METABOLISM OF THE
MEALWORM.

At 23° C., when mealworms are starved at various humidities, they retain their original ratio of water to dry matter (Buxton, 1930); but they lose weight at a different rate at each humidity, and it has been shown above that the rate of loss of weight is roughly proportional to the saturation deficiency of the air (p. 387). Thus the rate of metabolism is different at each humidity, and it is regulated so as to produce sufficient water of metabolism to maintain the proportion of water and dry matter constant.

At 30° C., except in dry or very moist air, the rate of loss of water is also proportional to the saturation deficiency; but the rate of metabolism of the mealworm is also increased by the higher temperature, and a larger amount of metabolic water is produced. Fig. 1 shows the proportions of dry matter and water in the mealworms before and after starvation. It will be seen that, after starvation at 30° C. in atmospheres of different humidities, they all contain the same amount of dry matter; their rate of metabolism has been the same in each case as they have used up the same amounts of food reserves. But the loss of water has been proportional to the saturation deficiency of the air at 30 and 60 per cent. relative humidity, and although they have lost water as fast as they can, yet the proportion has risen because so much metabolic water has been formed. At 23° C. the mealworms quicken their metabolism in dry air and decrease its speed in moist, but at 30° C. they cannot decrease the rate below a certain figure. At 37° C. the rate of metabolism is even more rapid, and in air with a relative humidity of 70 per cent. the insects become quite "dropsical" in 14 days. At 8° C. the rate of metabolism was so low (the dry matter fell only 4 per cent. in six weeks) that it was impossible to draw any conclusions.

Mealworms are adapted to living in hot and dry air, or in cooler air at various humidities. In hot moist air their water balance is upset, as they are unable to get rid of their water of metabolism sufficiently rapidly. In very moist air they even take up water, and this upsets their internal conditions still further. Mealworms are able to conserve their water to a remarkable degree, but they have to rely on evaporation to get rid of superfluous water, and this arrangement breaks down when the air is too moist.

I am indebted to Dr. P. A. Buxton for his help in this work, and for reading the manuscript; to Dr. M. Goldblatt for assistance in developing the technique

for the analyses ; and to Dr. J. S. Haldane, F.R.S., for reading and criticising the manuscript.

SUMMARY.

1. The loss in weight of starving mealworms under controlled conditions of temperature and humidity was studied.

2. Analyses of the insects before and after starvation were made, to show what food reserves were used. The mealworm used mainly fat, and in addition some carbohydrate (principally glycogen) and protein.

3. The quantity and nature of the food reserves used was such that the weight of metabolic water produced almost exactly balanced the loss of carbon. This means that if these insects do not excrete during starvation, any loss in weight is due to evaporation of water.

4. The rate of loss of water from mealworms was found to be roughly proportional to the saturation deficiency of the air at 8° C., 23° C., 30° C. and 37° C., and at most humidities. In very hot dry air they conserve their water. In very moist air, with 90 per cent. relative humidity, mealworms take up water from the atmosphere at 8° C., 23° C., or 30° C. This phenomenon depends on the relative humidity, not the saturation deficiency, and so resembles the hygroscopy of inanimate objects.

5. At 23° C. the mealworms regulate their rate of metabolism (and their production of metabolic water), so keeping a constant ratio of water to dry matter in their bodies. At 30° C. the rate of metabolism is higher, and the same at all humidities. The rate of loss of water is proportional to the saturation deficiency, but in moist air water is lost less rapidly than it is produced by metabolism, and the insects become dropsical.

6. The Tenebrionid beetles are adapted to hot, dry conditions, and their water balance is upset when they are starved in moist air.

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The Independence of Vitamin B₁ Deficiency and Inanition.

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(From the Department of Biochemistry, Oxford.)

(Communicated by Sir Charles Sherrington, F.R.S.—Received June 30, 1932.)

The object of this communication is to prove finally that the nervous symptoms in vitamin B₁ deficiency and the change in oxidative behaviour of pigeon's brain tissue are intimately related and are entirely independent of loss of weight, or general inanition. Previous work (Gavrilescu and Peters, 1931) had shown that the brains or tissues of birds after complete cure had a normal oxidative behaviour in glucose-phosphate solutions, at a time when no increase in weight had taken place. This left little escape from the conclusion that the symptoms were not due to the inanition. Nevertheless, the point was felt to be so important that further proof was desirable, with even more rigorous control. An opportunity for this was presented by the recent discovery (Gavrilescu, Meiklejohn, Passmore and Peters, 1932) of the essential importance of lactate rather than glucose, and of the large *in vitro* effect of vitamin B₁ concentrates. Accordingly, we have followed the oxygen uptakes in lactate solutions of the brains of pigeons which have been cured of opisthotonus symptoms by dosing with vitamin B₁ concentrates. A marked increase in the oxygen uptake has been found corresponding with the disappearance of the nervous symptoms, and the restoration is accompanied by a decrease in the effect of the concentrates *in vitro*.

Methods.—These have been as previously described, and the figures quoted in the tables are calculated from the oxygen uptakes at 38° C. of 50–100 mg. of the minced brain tissue, suspended in 3·0 c.c. lactate-phosphate solution. The vitamin B₁ concentrate had an activity for the bird of approximately 0·05 mg.,‡ and the same concentrate was used throughout, both to cure and in the bottles *in vitro*. The vitamin B₁ concentrates have been given both orally and intramuscularly; symptoms usually disappear within 3 hours in the former case, and 1 hour in the latter. No food was given after dosing, and the

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† Senior Scholar of Brasenose College.

‡ Pigeon day don.

amounts of water in the case of the lightly cured birds has been limited to 5–15 c.c.* The lactate used for these experiments was made as before from zinc lactate and put up in sterile tubes in amount sufficient for each experiment, the tubes being sterilised by heating on three successive days and thereafter kept in cold store.

Results.—Two sets of determinations have been made; one with birds killed as soon as they have come out of symptoms, and the other with birds which have been allowed a longer time to recover after dosing. In the former case the birds had not usually recovered sufficiently far to see properly. Table I shows the figures from 12 experiments with, and without, the addition of vitamin extract. In figs. 1 and 2 these are compared with similar sets of observations from normal and avitaminous birds.

Table I.
(a) Birds Just Out of Symptoms.

Experiment.	How dosed.	Hours between dosage and death.	Cerebrum.		Optic lobes/ rest.	
			Contr.	Vit.	Contr.	Vit.
291	Intramuscularly, 8 doses	$\frac{3}{4}$	1430	(1530)	995	1180
290	Intramuscularly, 8 doses	1	1325	1570	1100	1395
274	Intravenously, 8 doses	1	1350	1920	1225	(1355)
284	Orally, 12 doses	2	1380	1530	1270	(1390)
273	Intramuscularly, 8 doses ..	$2\frac{1}{4}$	1520	1770	(1220)	1320
285	Orally, 8 doses	$3\frac{1}{2}$	(1430)	—	1030	1420
Average			1410	1665	1140	1340
Standard deviation of the mean			28	76	46	37

(b) Birds Dosed and Well Recovered.

283	*Orally, 12 doses	3	1665	1655	(1381)	—
292	Intramuscularly, 8 doses	5	1540	1585	(1295)	1485
272	Orally, 6 doses	18	(1480)	(1560)	(1350)	(1440)
277	Orally, 8 doses	18	1685	1740	1420	1580
282	Orally, 8 doses	18	1660	(1680)	1330	(1540)
275	Orally, 8 doses ..	48	1650	(1800)	1405	1395
Average			1615	1670	1370	1490
Standard deviation of the mean			35	36	18	34

* 1 gm. of Marmite given in addition accidentally.

* We are grateful to Dr. S. Kon for stressing the need for controlling water intake.

In Table II and fig. 3, we have compared the average uptake for normal, birds well recovered, birds just out of symptoms and avitaminous birds, and also added the effect of the vitamin B₁ concentrates *in vitro*. Both parts of the brain show an improvement of 50 per cent. for the birds just out of symptoms, and normality for those in which the dose had been allowed to act for longer.

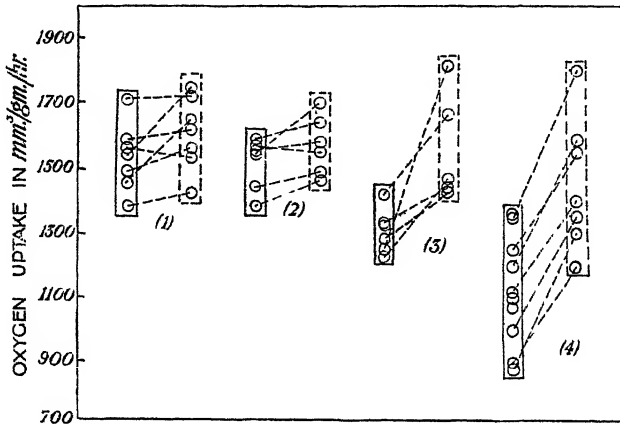


FIG. 1.—Oxygen uptake of cerebrums. Full line rectangles = lactate substrate; dotted line rectangles = lactate substrate + concentrate. 1, Normal; 2, Recovered well; 3, Just out of symptoms; 4, Avitaminous.

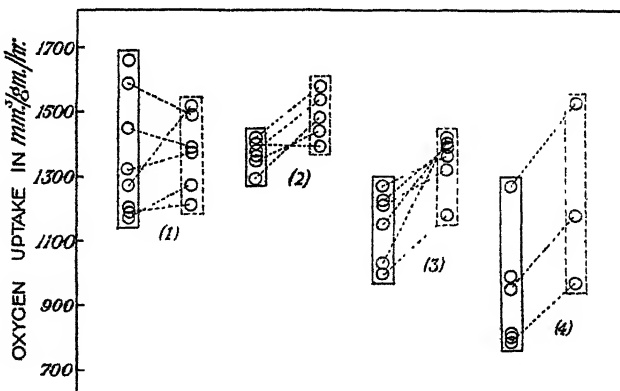


FIG. 2.—Optic lobes/rest. Full line rectangles = lactate substrate; dotted line rectangles = lactate substrate + concentrate. 1, Normal; 2, Recovered well; 3, Just out of symptoms; 4, Avitaminous.

The only addition to the avitaminous pigeon was the 0.4 mg. of solid weight given with the vitamin B₁ concentrate and water. In such a bird, water alone does not cure the symptoms. Hence, the changes restoring normality in symptoms and oxidative behaviour must be directly due to the administration

Table II.—Comparison of the Oxygen Uptakes of Avitaminous, Dosed Avitaminous and Normal Pigeon Brain (Substrate Lactate), Averages.

Bird.	Cerebrum.				Optic lobes/rest.			
	Control.	S.D. of mean.	Vitamin addition.	Per cent.	Control.	S.D. of mean.	Vitamin addition.	Per cent.
Avitaminous	1220	53	1560	28	935	68	1230	22
Just out of symptoms	1410	28	1665	17	1140	46	1340	19
Recovered well	1615	35	1670	3	1370	18	1490	9
Normals	1620	46	1710	4	1380	63	1380	0

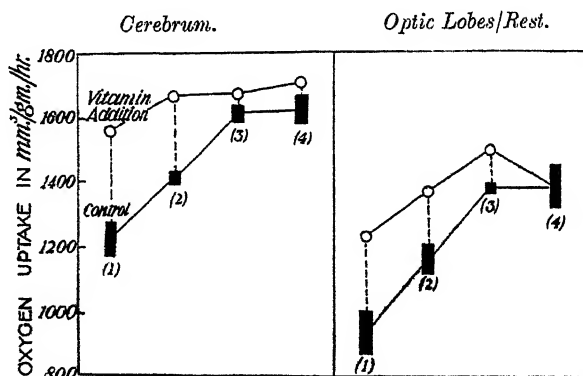


FIG. 3.—1, Avitaminous; 2, Just out of symptoms; 3, Recovered well; 4, Normal. The black rectangles represent the standard deviation of the mean of the observations.

of the vitamin B₁ concentrate—an amount not exceeding 1/500,000 of the body weight of the pigeon. It is felt that these experiments exclude absolutely the view that the essential symptoms are due to general inanition rather than to vitamin deficiency, a question so often discussed (Abderhalden and Vlasiopoulos, 1931; Galvao and Cardoso, 1930). Of course it is not implied that some of the disturbances found in avitaminous birds are not common to starvation, but that superimposed upon these there are biochemical lesions in the brain specific to the vitamin, and associated both with accumulation of lactic acid (Kinnersley and Peters, 1930) and change in oxidative behaviour.

Note.—It is unlikely that the cerebrum itself is concerned with the symptoms. The arguments previously advanced for their relation to the lower parts of the brain are still valid. After dosing with vitamin B₁ the restoration of oxidations appears to take place simultaneously all over the brain. The delay

required for complete recovery is not surprising when it is remembered that diffusion to the active surfaces of the cell is presumably necessary.

Summary.

The brains of avitaminous birds dosed with very small amounts of vitamin B₁ concentrates show an improvement in oxidative behaviour *in vitro*, in presence of lactate, corresponding with disappearance of the nervous symptoms. The latter are specifically related to oxidation changes induced by the vitamin deficiency and are independent of the general state of the nutrition.

We are indebted to Mr. H. W. Kinnersley for the preparation of the concentrate used in these experiments, also to the Government Grant Committee of the Royal Society and to the Medical Research Council for grants towards the cost of the experiments.

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Denaturation of Proteins in Muscle Juice by Freezing.

By D. B. FINN.

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(Communicated by Sir William Hardy, F.R.S.—Received June 16, 1932.)

During an investigation on the freezing point curve of ox muscle juice, it was observed that upon thawing a sample which had been held in the frozen state at -3° C. for some time, a flocculent precipitate of protein formed. Further experiments showed that the precipitation depended upon the conditions of freezing. A search of the literature yielded little information regarding the phenomenon, practically the only investigation bearing directly on it being that of Vickery (1926), who, though he did not observe precipitation, states that the number of particles visible under the ultra microscope diminished after freezing and thawing, owing, as he thought, to an aggregation of protein particles.

The precipitated protein was found to be insoluble in salt solutions, and was, in fact, denatured. The circumstances of this denaturation are interesting, for nothing more is involved than the removal of water from the system by freezing, and as this factor can be quantitatively controlled, the system affords an excellent opportunity for studying the mechanism of denaturation.

In any investigation on the effect of freezing it is necessary, as far as possible, to separate two factors—the rate of freezing and the degree or severity of freezing.

In the present investigation variation of the first factor, rate of freezing, was eliminated by freezing the juice rapidly in a bath at -20° C., and subsequently warming it rapidly to the required temperature of storage. It will be shown in the paper that rapid freezing followed by rapid thawing causes no sensible denaturation of the proteins in muscle juice.

The investigation has included an examination of:—

- (1) The extent and rate of denaturation at different temperatures in the frozen state;
- (2) The hydrogen ion and salt concentration of frozen muscle juice;
- (3) The influence of hydrogen ion and salt concentration on denaturation.

The data must be regarded as being preliminary in nature. They will be followed by more detailed studies, especially in regard to the protein fractions concerned and their relation to those in the muscle before the onset of rigor.

I.—THE EXTENT AND RATE OF DENATURATION AT DIFFERENT TEMPERATURES OF FREEZING.

The Effects of Rapid Freezing and Thawing.

The juice was obtained by grinding lean beef with twice its weight of sand and pressing it through canvas at about twenty tons pressure. It was then centrifuged to remove suspended particles. The initial freezing points of samples thus obtained varied between -1.05°C. and -1.08°C. , the total nitrogen from 1.4 to 1.9 gm. per 100 c.c., the total coagulable nitrogen (by precipitation with trichloroacetic acid) from 0.98 to 1.2 gm. per 100 c.c., and the p_{H} from 5.5 to 5.8 as measured by the glass electrode at 18°C.

Samples of juice in well-stoppered test tubes of 2 cm. diameter were placed in a bath of rapidly circulating brine at -20°C. A cooling curve showed that the temperature at the centre of a tube reached -20°C. in ten minutes, but in actual fact the tubes were left in the bath for two hours to insure uniformity of temperature throughout the samples. They were then thawed in running water, after which known volumes were centrifuged at 6000 r.p.m. for 30 minutes. Only a slight precipitate settled out, which, when examined under the microscope, was found to be crystalline in nature and proved to be principally creatine.* Centrifuging at a higher rate of speed for longer times failed to bring down more than traces of protein.

Similar experiments were carried out on samples of muscle juice frozen in solid carbon dioxide and ether at -72°C. , and thawed rapidly in running water; the same result was obtained, only a very slight precipitate settled out, amounting to not more than 1 per cent. of the total coagulable nitrogen. This observation, that rapid freezing in brine at -20°C. (or solid carbon dioxide and ether at -72°C.), followed by rapid thawing, produced no sensible change in the state of the proteins of muscle juice, formed the basis of the work, which immediately follows, on the effects of different temperatures of storage on the extent of denaturation. In each case the sample of juice was frozen in the cold brine and then transferred rapidly to a brine held at the temperature under investigation. At the end of the required period of storage the sample was thawed rapidly in running water. In this way the change in state of the proteins at the completion of a freezing-storage-thawing cycle could be referred with certainty to storage for a definite time at a definite temperature; it was

* The separation of creatine and also creatinine actually commences at a temperature of about -3°C.

not complicated by the changes at other temperatures, such as would have occurred if the samples had been frozen slowly and/or thawed slowly.

The Effect of Rapid Freezing and Subsequent Storage.

A series of tubes containing the same juice were frozen in brine at -20°C . and placed in constant temperature rooms which were controlled to within $\pm 0.2^{\circ}\text{C}$. and held at various temperatures between -1.5°C . and -20°C . At specified intervals between 10 and 80 days the tubes were taken out of storage, thawed rapidly in running tap water at about 18°C ., and slowly shaken for two hours at room temperature. This was done (a) to re-dissolve substances which had been thrown out of solution during freezing merely by their limit of solubility being exceeded, and (b) to re-dissolve the protein if reversible precipitates were present, a matter which is discussed later. Measured quantities, usually 20 or 25 c.c. of the juice, were then centrifuged. and the precipitates washed twice by suspending them in 0.01 M phosphate buffers at p_{H} 6.6 containing 0.03 M KCl, and again centrifuging. This procedure was adopted since it was found that certain of the precipitates which occurred in those tubes stored above -3°C . were peptized or dissolved by distilled water, but were not so affected by the solution described. The nitrogen in the precipitate was determined by the Kjeldahl method.

The results of two typical experiments are shown in figs. 1 and 2. It will be observed that denaturation is greatest at the higher temperatures of storage. In fig. 1 it will also be observed that the *rate* of denaturation is greater at -3°C . than at -1.5°C . A more detailed study at temperatures just below the freezing point showed that the rate of denaturation was a maximum at about -2° to -3°C . This is shown in fig. 3, which records the extent of denaturation in samples held at temperatures from -1.2° to -20°C . for 12 and 30 days.

Apparently only a fraction of the protein is readily denatured. At -3°C . some 20 per cent. of the protein is denatured at a comparatively rapid rate in 40 days. Subsequent denaturation is slow; in the case of juice No. 6 (fig. 1), no further denaturation was observed after 35 days. This would appear to indicate that the easily denatured protein constitutes a separate fraction of the protein of the juice.

There is a good deal of variation between samples, and more evidence will be necessary before any conclusions can be arrived at regarding the difference in behaviour suggested in figs. 1 and 2 of a muscle juice prepared 24 hours

after death and one prepared nine days after death. From the present work the only point of difference seems to be that equilibrium is attained more

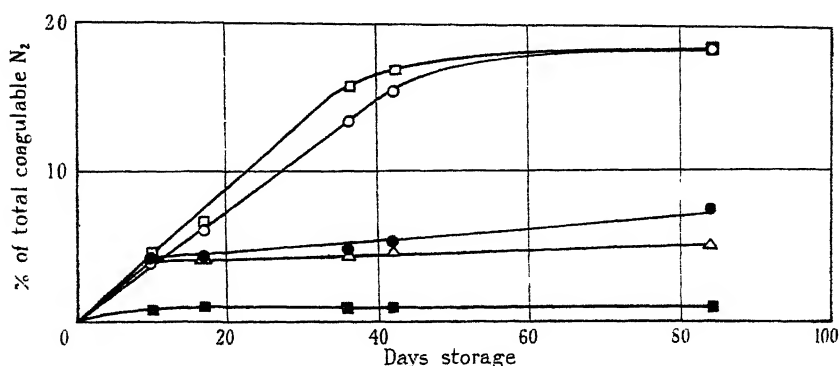


FIG. 1.—The influence of time and temperature of storage upon the precipitation of proteins in ox muscle juice.

Beef juice No. 6; T.C.N. 1.07 per cent.; initial p_H 5.80; prepared 24 hours after death.

○ Stored at -1.5°C .; □ at -3.0°C .; ● at -6.0°C .; △ at -10.0°C .; ■ at -20.0°C .

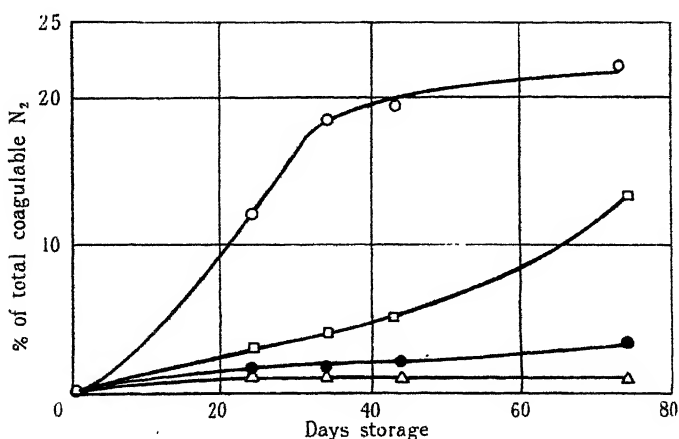


FIG. 2.—The influence of time and temperature of storage upon the precipitation of proteins in ox muscle juice.

Beef juice No. 5; T.C.N. 1.03 per cent.; initial p_H 5.75; prepared 8 days after death.

○ Stored at -2.5°C .; □ at -6.0°C .; ● at -10.0°C .; △ -20.0°C .

slowly in the older juice—the amount of denatured protein at -3°C . is apparently still increasing even after 75 days' storage.

The Reversibility of the Protein Precipitates.

In order to make sure that the precipitates were not merely proteins salted out by the concentrated salts in the frozen muscle juice, a comparison was made between the precipitates which could be obtained from two sets of

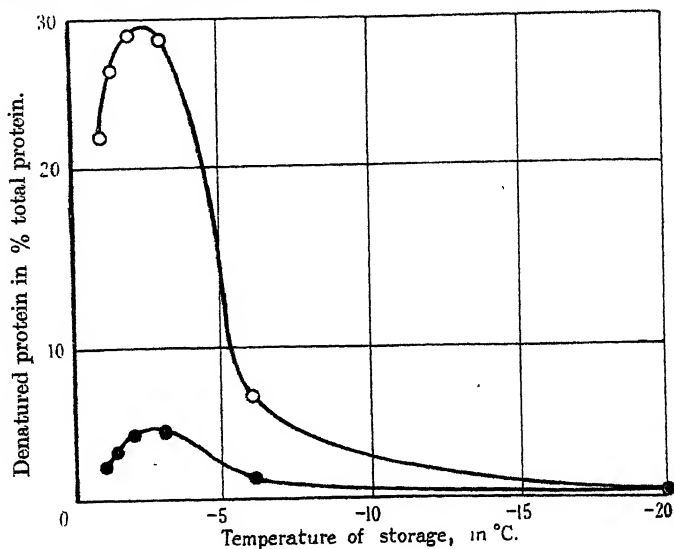


FIG. 3.—Extent of denaturation of protein in muscle juice when stored at temperatures between -1.2 and -20°C. for 12 and 30 days.

Beef juice No. 10; T.C.N. 1.217; initial p_{H} 5.62; prepared 9 days after death.

● Stored for 12 days; ○ Stored for 30 days.

samples from the same muscle juice which had been stored for 42 days at various temperatures. One of these sets was centrifuged immediately after thawing, the other agitated slowly for three hours at 18°C. before being centrifuged. The results are given in Table I, and show that no appreciable

Table I.

Temperature of storage.	Per cent. of total coagulable nitrogen in precipitate from juice.		Per cent. re-dissolved.
	Immediately after thawing.	Thawed and agitated for 3 hours.	
-1.5°C.	12.9	14.8	— 2.1
-3°C.	16.2	15.4	0.8
-6°C.	4.8	4.5	0.3
-10°C.	4.9	4.6	0.3
-20°C.	0.6	0.5	0.1

resolution of the precipitates took place during three hours' agitation in the mother liquor.

It was concluded, therefore, that the precipitates were denatured proteins, using the word "denatured" in the sense that Wu (1931), for example, uses it, namely, that a protein is denatured when it has undergone such change as to be insoluble in solutions in which it was formerly soluble. Precipitates from other samples of frozen muscle juice were found to be insoluble in buffers from p_H 3 to p_H 8 and of molar concentrations varying from 0.3 to 0.05, but were sometimes re-dissolved by higher molarities at the lower hydrogen-ion concentrations. They were, however, dissolved by N/14 sodium hydroxide, which is characteristic (Lewis, 1931) of many denatured flocculated proteins.

The Effect of Prolonged Storage at -20° C. upon Subsequent Denaturation at -3° C.

It was thought that prolonged storage in the frozen state at -20° C. might have the effect of making the proteins less stable, and more easily denatured at higher temperatures. To test this a muscle juice which had been stored for 42 days at -20° C. was placed in -3° C. Examinations were made at

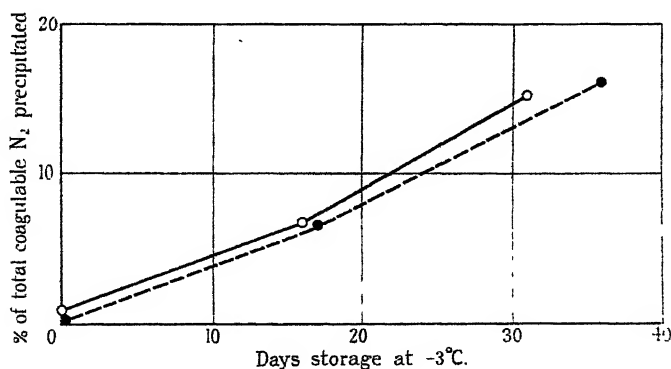


FIG. 4.—The effect of storage at -20° C. and subsequent storage at -3° C.

○ Stored at -20° C. for 40 days prior to storage at -3° C.; ● the same muscle juice without preliminary storage at -20° C.

intervals, and the rate of denaturation compared with that of the same muscle juice at -3° C. without previous storage at -20° C. The results are shown in fig. 4, and it can be seen that there is but slight difference in the rate of denaturation; the proteins concerned, therefore, are little changed by freezing and storage at -20° C. for 42 days.

II.—THE HYDROGEN-ION CONCENTRATION OF FROZEN MUSCLE JUICE.

The increased concentration of inorganic salts in the aqueous phase of frozen muscle juice might be expected to change the hydrogen-ion concentration of the juice. Such a change would affect the denaturation and precipitation of proteins. Measurements have therefore been made of the p_H of muscle juice stored at various temperatures for various periods, and of the concentrated solutions which separate from juice frozen at various temperatures. Attempts to measure directly the p_H of the frozen juice failed owing to the variations which occurred in the potential developed in the glass electrode during and subsequent to freezing. This, however, did not occur in the absence of freezing, and measurements of the change of p_H between 0°C. and -5°C. in a concentrated (F.P. -6.28°C.) sample were made.

The Effect of Temperature on Hydrogen-ion Concentration.

For these measurements decinormal hydrochloric acid was used on the outside of a membrane of low resistance glass, and the electrode calibrated by measuring the electromotive force produced when known buffers, slightly more acid and slightly more alkaline respectively than the juice to be examined, were placed on the inside. The change in p_H of the buffers with temperature was followed by means of the hydrogen electrode, assuming that the p_H of decinormal hydrogen chloride had a constant value of 1.08 over the temperature range used. The glass electrode, calomel cells, buffers and muscle juice were all in thermal equilibrium at the various temperatures examined. The results are set forth in Table II.

Table II.—The Change in p_H between $+15.0^\circ \text{C.}$ and 0°C. in Ox Muscle Juice, prepared after Rigor.

Juice.	p_H at 15°C.	p_H at 0°C.	Change per 0°C.
A	5.70	5.99	— 0.0193
B	5.65	5.96	— 0.0206
C	5.55	5.83	— 0.0186
D	5.62	5.93	— 0.0207
	Mean value.		— 0.02

A sample of juice was also concentrated by evaporation until its freezing point was -6.28°C. This sample at 0°C. had a p_H of 5.40, at -5°C. its p_H was 5.50, i.e., a change of -0.020 p_H per degree C.

From these results it can be seen that as the temperature is lowered the p_H increases in the order of 0.02 units per degree C. Thus, in a muscle juice of p_H 5.5 at 18° C., the hydrogen-ion concentration in the same juice when super-cooled would be about p_H 5.9 at — 3° C. and p_H 6.0° at — 10° C. When freezing commences, however, concentration of the aqueous phase takes place, producing a further change in p_H .

The Effect of Removal of Water on Hydrogen-ion Concentration.

In order to measure such changes, a large quantity of muscle juice was frozen to equilibrium to a little below its freezing point, and the remaining aqueous phase drained away from the ice. The temperature of the fluid was allowed to rise in order to melt small fragments of ice which had escaped filtration; the freezing point and water content were then accurately determined, and the p_H measured at 18° C. These operations were repeated at progressively lower temperatures, until at — 10° C. the product was too small in bulk and too viscous for further treatment. Fig. 5 shows the percentage of water withdrawn as ice from muscle juice at temperatures down to — 15° C. The curve is practically identical with the curve of water removal from a 0.333 M potassium chloride solution which is isotonic with the juice.

Fig. 6 shows the change in p_H of the liquid phase of frozen juice as the temperature is lowered. In constructing this curve the temperature coefficient (previously determined) of 0.02 p_H units per degree C. was employed. It will be observed that the removal of water up to 78 per cent. is accompanied by a change to the acid side of the original p_H , and that further concentration leads to a shift to the alkaline side. Thus, when 80 per cent. of the water has been removed, there is a p_H change of from + 0.1 to + 0.2. At this concentration the freezing point of the juice is — 6° C. By referring to figs. 1, 2 and 3 it will be seen that at and below this temperature denaturation is markedly diminished. In the next section it is shown that the two observations are intimately connected.

III.—HYDROGEN-ION CONCENTRATION AND DENATURATION.

Buffers of constant concentration of 1 M per 1000 c.c., but of varying p_H , were prepared using the data of Cohn (1927) and Cohn, Heyroth and Menkin (1928). 10 c.c. of these buffers were then added to a series of tubes, each containing 25 c.c. of freshly prepared muscle juice, making a total molarity of 0.5. These were frozen in rapidly circulating brine at — 20° C. and stored

at -3°C . Since the temperature of storage was below the freezing point of the juice, the total molar concentration of salts to which the proteins were exposed during storage would be identical with that of untreated juice at that temperature; the proportions of the different ions, however, would be different. At the end of 24 days the tubes were thawed and the p_{H} measured at 18°C .

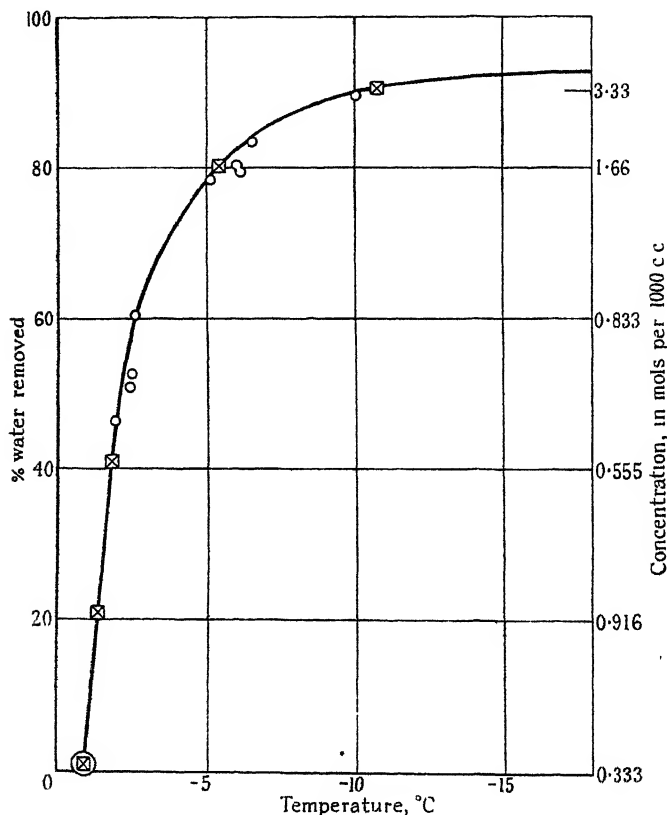


FIG. 5.—The water removed by freezing to various temperatures.

○ Muscle juice; ☒ 0.333 M KCl.

The precipitates were centrifuged off, and washed with buffers of the same p_{H} and molar concentration as the juice-buffer mixtures. The nitrogen in the precipitates was estimated, and a curve constructed showing the relation between the p_{H} and the percentage of total coagulable nitrogen precipitated. The data are set forth in fig. 7.

The effect of hydrogen-ion concentration is very marked. From p_{H} 7 to p_{H} 6 the denaturation is at a constant low level of approximately 1 per cent.

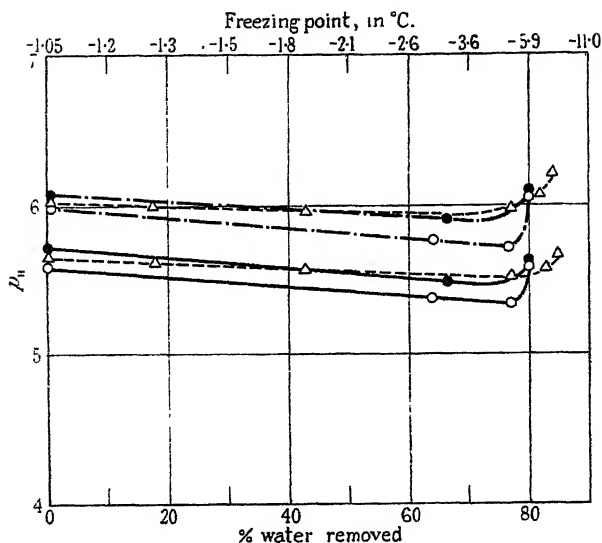


FIG. 6.—Changes in hydrogen-ion concentration produced by removal of water from muscle juice.

Upper curves : p_H at freezing point.

Lower curves : p_H at 18°C .

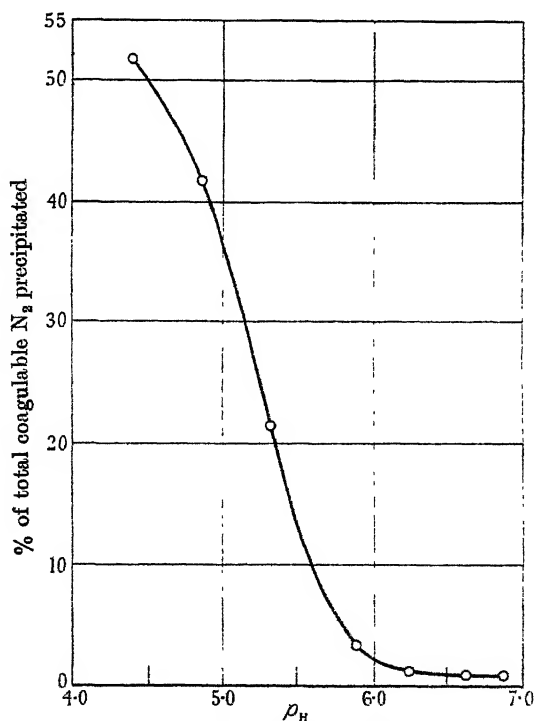


FIG. 7.—The effect of hydrogen-ion concentration on the denaturation and precipitation of proteins in ox muscle juice.

On the acid side of p_H 6 there is a sharp increase. Between p_H 6 and p_H 4.8 denaturation increases regularly, until at p_H 4.8 40 per cent. of the coagulable nitrogen is precipitated.

These results suggest a partial explanation of the effects of temperature of storage on the extent of denaturation in frozen muscle juice. Between the freezing point and about -6° C. the p_H of frozen muscle juice is on the acid side of p_H 6.0, in which region, as the above experiments show, considerable denaturation is to be expected. Below -6° C., where the p_H rises again to 6 and higher, denaturation is very slight. The peak of denaturation between -2° and -3° C. is, however, not explained.

IV.—SALT CONCENTRATION AND DENATURATION.

Fresh muscle juice contains approximately 90 per cent. of water. The evidence available indicates that practically all this water is free to dissolve the inorganic constituents present in the juice.

The mean freezing point of the samples of juice examined was -1.1° C., which corresponds to that of a solution of potassium chloride 0.333 M. If the degree of drying of muscle juice (expressed as per cent. water removed) be plotted against the freezing point of the juice, the curve in fig. 5 is obtained. This is identical with the curve derived from a 0.333 M solution of potassium chloride. From this it follows that no appreciable separation of any constituent other than water occurs, so that the salt environment of the proteins at any temperature in the frozen state can therefore be calculated if the concentration of salts in the unfrozen juice is known. The following is the mean of analyses of four samples of ox muscle juice (Smith):—

	Per cent. of juice.						
K ⁺	0.52
Na ⁺	0.063
Ca ⁺⁺	0.023
Mg ⁺⁺	0.023
Phosphate (P) ⁻⁻⁻	0.20
Cl ⁻	0.08
Lactate ⁻	1.10
Nitrogenous bases (N) (mainly creatine and carnosine)							0.57

It will be seen that the ions present in muscle juice, apart from that of protein, are preponderatingly potassium, phosphate and lactate ions. In investigating the effect of increased salt concentration at constant hydrogen-ion

concentration, attention was only paid to the potassium and phosphate ions, data for these being readily available. No attempt was made to allow for the alteration in the concentration of lactate ion.

The rôle of salt concentration in the denaturation which occurs during freezing and storage was investigated in the following manner. Mixtures of solid potassium di-hydrogen phosphate and di-potassium hydrogen phosphate were dissolved in samples of muscle juice in such a way that the molar concentration varied while the p_H remained constant. Two series of experiments were conducted at p_H 5.2 and 6.0 respectively.

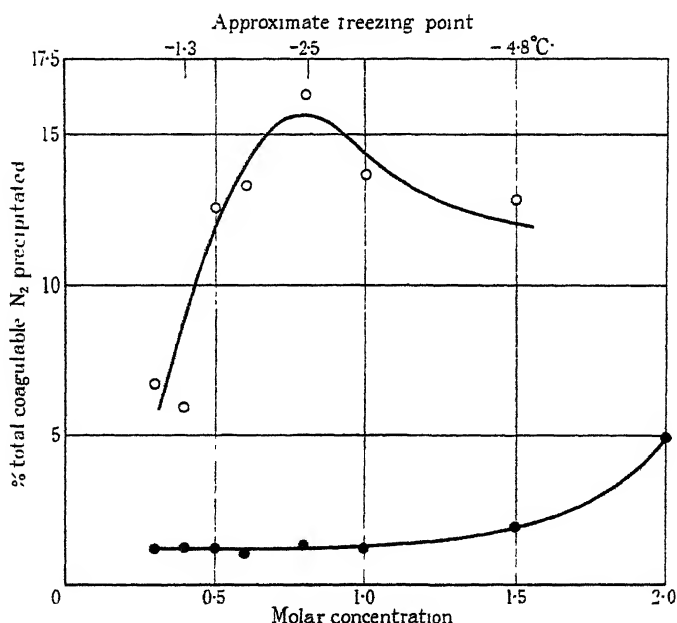


FIG. 8.—The effect of increasing concentration of phosphates at p_H 5.2 and p_H 6 upon denaturation of proteins in ox muscle juice at 0°C . \circ p_H 5.2; \bullet p_H 6.0.

The dissolved mixtures were stored at 0°C . for 15 days. They were then diluted with distilled water, bringing the molar concentration to 0.33 M per 1000 c.c. (the concentration of untreated muscle juice), in order to simulate the process of thawing. The precipitates were separated by centrifuging, and were washed with 0.33 M buffers at the same p_H as the samples. The data are presented in fig. 8. It will be seen that at p_H 6 the denaturation occurring at molar concentrations from 0.4 to 1.0 is in the order of 1 per cent. of the total coagulable nitrogen; at higher concentrations there is a gradual increase,

but at 1.7 molar (equivalent to the concentration of salts in the liquid phase at $-6^{\circ}\text{C}.$) the denaturation is still only of the order of 3 per cent. of the total coagulable nitrogen. At p_{H} 5.2 the denaturation is much greater and comes to a maximum at about 0.8 mols. This coincides with the molar concentration of untreated muscle juice which is frozen to $-2.5^{\circ}\text{C}.$, the temperature at which maximum denaturation is actually observed.

Discussion.

Denaturation occurs in ox muscle juice when it is stored at various temperatures below its freezing point. With fresh muscle juice the denaturation reaches a maximum after a period of 25 to 40 days at -2° to $-3^{\circ}\text{C}.$, and amounts to approximately 20 per cent. of the total coagulable nitrogen. Storage up to 80 days at these temperatures does not produce any sensible increase in precipitation. This seems to indicate that there is one fraction of the protein which is more easily denatured than the others.

The mechanism of denaturation of the proteins in the frozen juice seems clear. There is a sharply defined critical point in the hydrogen-ion concentration which lies at p_{H} 6 (fig. 7). On the acid side of this there is a marked and rapid increase in denaturation, but on the alkaline side denaturation is relatively slight. It has also been shown, fig. 8, that on the acid side of p_{H} 6 there is a certain salt concentration at which maximum denaturation occurs; at p_{H} 5.2 this is 0.8 M per 1000 c.c.

By referring to figs. 5 and 6 it can be seen that in the zone of maximum denaturation—that is, at -2° to $-3^{\circ}\text{C}.$ —the salt concentration is about 0.8 M, and the hydrogen-ion concentration is on the acid side of p_{H} 6. These two factors, acting together, are responsible for the denaturation which is actually observed. At temperatures below $-3^{\circ}\text{C}.$, where less denaturation occurs, it will be seen that the hydrogen-ion concentration is moving to the alkaline side of p_{H} 6, and the salt concentration, now greater than 0.8 M, is also increasing. Both of these changes reduce denaturation. Thus at $-6^{\circ}\text{C}.$ the p_{H} has shifted to between 6.1 and 6.2, and the salt concentration to about 1.7 M per 1000 c.c. It can be said, therefore, quite definitely, that it is the variation in the two factors, hydrogen-ion concentration and salt concentration, brought about by a reduction in temperature and removal of water, that is responsible for the observed denaturation.

Little is known regarding the proteins contained in muscle. Von Fürth (1919) regarded them as being (1) soluble myogen fibrin, probably a denatura-

tion product of myogen which was precipitated by dialysis and by half saturation with ammonium sulphate and coagulated between 10° and 40° C.; (2) myosin, which was precipitated by dialysis and by one-third saturated ammonium sulphate and coagulated between 47° and 50° C.; (3) myogen, which was not precipitated by dialysis, but was precipitated completely by one-half saturated ammonium sulphate and coagulated between 55° and 60° C. Halliburton (1887) found three protein fractions in the salt extracts of fresh muscle, using as a method of identification heat coagulation in salt solutions. He did not find soluble myogen fibrin, but it seems probable that his paramyosinogen and myosinogen are identical with the myosin and myogen of Von Fürth. Good agreement between the two investigations is hardly to be expected, since little was known at the time regarding the influence of hydrogen-ion concentration or of salt concentrations upon denaturation, factors which must have an effect in determining the presence or absence of "soluble myogen fibrin" in any muscle extract. An attempt to repeat Von Fürth's separation with ammonium sulphate upon muscle juice disclosed the fact that only 50 per cent. of the total protein was removed by half saturation with ammonium sulphate, leaving another fraction which became progressively more insoluble as the salt concentration was increased. When this later fraction was re-dissolved by dilution, it was found that upon half saturating it once more with ammonium sulphate, flocculation occurred. These fractions are therefore by no means so well defined as might be supposed from Fürth's account.

Edsall (1930) has isolated and described the physical and chemical properties of one of the fractions, myosin. He shows that ox myosin is relatively insoluble at salt concentrations below 0.3 M at all p_H values. Smith (unpublished data) finds that at p_H 5.6 the solubility of rabbit myosin first increases and then decreases with increase in concentration of potassium chloride solutions. It is almost insoluble in 0.2 M and 1 M solutions, and has a maximum solubility of 140 mg. per cent. in 0.6 M solutions.

There can, then, be only slight amounts of myosin in the muscle juice used, which had a hydrogen-ion concentration of from p_H 5.5 to p_H 5.8 and a freezing point depression of -1.08° (0.3 M). Dilution of fresh muscle juice with distilled water precipitates not more than 3 per cent. of the total coagulable nitrogen, and this fraction in all probability consists mainly of soluble myogen fibrin.

It seems evident that the fraction of muscle juice which is denatured by freezing cannot be myosin, but must be a part of the Von Fürth myogen

fraction. The question of its identity and the relation of the protein fractions in expressed muscle juice, prepared after rigor, to those in the living muscle will be made the subject of a later paper.

There seems to be a definite relation between denaturation of proteins by freezing and the quantity of "drip" (a term used to describe the fluid which escapes on thawing from muscle which has been frozen).

Moran (1932) finds that this drip reaches a maximum when frozen tissues are stored at from -2° to -3° C. for 80 days. He has shown that at -1.5° C. and at temperatures lower than -3° C. there is a diminution of the effect. This is in harmony with the extent of denaturation, as may be seen by referring to figs. 1, 2 and 3.* Denaturation during freezing may also help to explain the results of Fearon and Foster (1922), who showed that slow freezing increased the rate of autolysis in beef tissue, it being known that denaturation renders proteins more susceptible to the action of enzymes (Talarico, 1910, and Lin, Wu and Chen, 1928).

Summary.

(1) The rate and extent of denaturation of proteins in frozen muscle juice, expressed after the onset of rigor, have been determined. The rate is greatest when the frozen juice is held between -2° and -3° C., in which zone a maximum of from 25 to 30 per cent. of the total protein is denatured after about 40 days. At these temperatures storage for longer periods up to 80 days does not result in a sensible increase of denaturation. It therefore seems that one fraction of the proteins contained in muscle juice is more easily denatured than are the others. Evidence is adduced that this is part of the myogen fraction of Von Fürth.

(2) The changes in salt and hydrogen-ion concentration during freezing have been investigated, as well as the influence of these two factors upon denaturation of the proteins in muscle juice. The maximum denaturation at from -2° to -3° C. can be explained by the variation of these two factors in the frozen muscle juice.

* If there is an intimate relationship between "drip" and denaturation it would appear that the former could be reduced by keeping the hydrogen-ion concentration of muscle in the frozen state at or above p_H 6. A possible method would be to perfuse the muscle with a phosphate buffer.

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The Osmotic Passage of Water and Gases through the Human Skin.

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It has been known for long that part at least of the "insensible" passage of water which is constantly passing through the skin is not due to the activity of sweat-glands, since in the rare cases of congenital absence of sweat-glands some water is nevertheless given off through the skin. Two cases of this kind were investigated by Loewy and Wechselsmann (1911), who inferred that at an ordinary air-temperature as much as 18 grams of water might pass through the skin in an hour. The cases were those of two brothers, whose father had apparently suffered from the same defect. A further, more complete, study of a similar case was recently made by Richardson (1926). In our previous paper (1929) we brought forward evidence to show that in ordinary insensible perspiration through the skin, salts do not penetrate the skin in anything like either the same concentration or the same composition as would be the case if they had been excreted in sweat, and several similar results were given by Moss (1927). Hence since sweat contains salt, and in fairly constant concentration, little or none of this insensible perspiration is due to sweating, and the passage of water is presumably due to an osmotic process. The present paper contains further evidence in this direction, together with data as to variations in the osmotic transference and their relation to regulation of body-temperature. Variations in the rate of transference of gases through the skin have also been studied.

We may define osmosis as the passage, molecule by molecule, of a substance, such as water, through a membrane or surface which is permeable to it, though it may not be to another substance present in the same state on one side of the membrane or surface. The consequence of the presence of the other substance is that the substance which can pass through the membrane can do so against adverse pressure on the side on which the other substance is present; and the maximum amount of this pressure is known as the osmotic pressure. As was pointed out recently by one of us (Haldane, 1928), this passage is due simply

to diffusion, the diffusion-pressure of the substance which passes through being necessarily greater on the side from which it passes, so that for this reason the substance can pass through against mechanical pressure. It is thus simply with a diffusion process that we are dealing in the case of osmotic transfer of water through the skin, and what is usually called the osmotic "pressure" existing in a solution is more correctly called osmotic depression. It is with this depression that depression of the vapour-pressure of the solution runs parallel.

If we regard insensible perspiration, or part of it, as an osmotic process it is not necessary to assume that the epidermis itself is a semi-permeable membrane. For if it were permeable to salts or other substances without sensible vapour-pressure, as soon as the concentration of any such substance on the epidermis became equal to that in the blood or lymph, the diffusion of that substance outwards would cease, while the diffusion of water outwards would proceed, since it would constantly be removed by evaporation so long as the dewpoint of the air was below the skin-temperature. A paper to appear later by Mr. H. Ramage and one of us seems, however, to indicate that the intact epidermis is actually impermeable to electrolytes in so far as diffusion is concerned.

If water passes through the skin by osmosis, a person in a bath of ordinary (not salt) water at about the skin temperature would be actually absorbing water slowly through the skin, while in a bath of sufficiently strong salt solution the skin would be passing water outwards at a considerable rate. We have therefore made experiments to test this point.

The same person (W.H.) was the subject of all the experiments. The procedure adopted was as follows. The subject was thoroughly sponged down from head to foot with ordinary tap water, dried, weighed, and then at once entered the bath, the water of which was not kept moving by any artificial means. In most of the experiments the subject left the bath every hour, and was rapidly dried and weighed. The usual duration of each experiment was six hours, the time taken for drying and weighing not being included. A sample of urine was taken prior to each experiment, and after each hour if any urine could be passed, the corresponding loss of weight being allowed for. No food or drink was taken during the course of an experiment. The usual type of weighing machine was found to be insufficiently sensitive, and all weighings were therefore made on a balance which had formerly been used for human metabolism experiments, and on which weighings to an accuracy of one gram can easily be made. The loss in weight due to metabolism and to evaporation of moisture from the lungs and throat was obtained experimentally during the

experiment, and when this was subtracted from the loss in weight measured on the balance, the net loss or gain in weight of the subject through the skin was obtained. The method of testing the absorption of water through the skin by observing the change in weight of an individual after immersion in a bath of water for some hours is subject to certain obvious sources of slight error; but in view of the actual results these are too small to be of much importance.

Since the Fahrenheit temperature-scale is in general use in English-speaking countries, the temperatures are stated in this scale. $5/9 (^{\circ}\text{F.} - 32) = ^{\circ}\text{C.}$

The results of all experiments are given in Table I. The bath temperature in experiments 1, 2, 5, 6, 7, 8 and 11 was kept at 91° – 93°F. , at which the subject remained just comfortable when the water was not stirred; and the mouth temperature remained normal throughout each experiment. There was thus no likelihood of any loss in weight by sweating. Experiments were also made with the subject naked (experiment 3) and ordinarily clothed (experiment 4) in the air of the room, for comparison with the bath experiments. In experiments 5, 6 and 7, the subject was wiped down with cleansing powder, ether, and soft soap respectively to remove any fat from the skin as far as possible, and so facilitate wetting. Bathing in salt water was repeated in experiment 8, and differed from experiment 1 in that the salt concentration was lower, and that the subject was washed with soft soap after each hour. In experiment 11, the bath consisted of 1.1 per cent. salt solution, this being approximately isotonic with blood-plasma. Hot baths at 99° – 100°F. were used in experiments 9, 10 and 12, and were undertaken chiefly to obtain data as to the amount and specific gravity of urine excreted during actual sweating in the bath.

The results of experiments 1 to 8 are shown graphically in fig. 1, where the loss or gain in weight through the skin is plotted against time. The salt-water baths gave a considerable loss in weight, approaching to the loss in the experiments in air, while the fresh-water baths gave on an average hardly any loss of weight. Since the loss in weight due to respiratory exchange had been deducted, the change in weight of the subject from start to finish of immersion in a bath would be nil if no absorption were taking place through the skin, and a gain in weight if net absorption were occurring. Under the experimental conditions, however, there would be a loss in weight from the head of the subject, which, of course, was not immersed, and a slight loss while the subject, after drying, was being weighed. But for this loss the extremely small net loss would certainly have been converted into a slight net gain.

The salt-water baths gave a loss in weight which clearly cannot have been due solely to evaporation from the subject's head, and indicate that water from the body was passing through the skin into the surrounding salt water by osmosis.

Any absorption of water through the skin in ordinary water baths might be facilitated by the removal of surface fat, but in the cases where this was carried out (experiments 5, 6 and 7) no sensible influence could be observed.

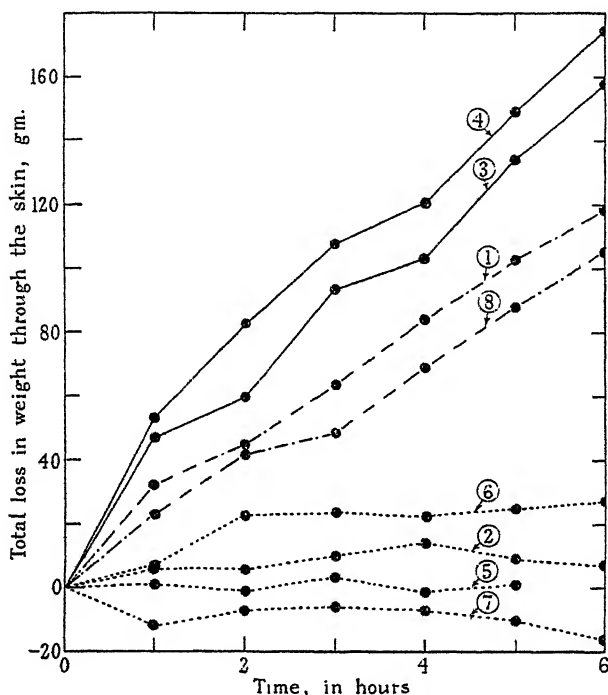


FIG. 1. ——— In air; - - - - Salt water bath; Ordinary water bath.

In experiment 11 the bath consisted of salt solution approximately isotonic with the blood, and the subject was weighed before and after the period of immersion, intermediate weighings at the end of each hour not being carried out. The total difference in weight of the subject was, however, of the same order as that in the ordinary water baths, and this is just what would be expected if loss from the skin is due to osmosis, since the osmotic depression of blood is very small.

The well-known swollen and furrowed appearance of the skin on the fingers and toes was noticed in the ordinary water and isotonic salt-water baths, but no signs of this were observed in the stronger salt-water baths (11.5 and 20

Table I.

Experi- ment No.	Kind of bath.	Period in hours.	Total measured loss in weight, grams.	Total loss in weight from lungs, grams.	Total loss in weight through skin, grams.	Average mouth tempera- ture per hour period, °F.	Average pulse rate per hour period.	Urine per hour period.		Remarks.	
								Grams.	Specific gravity.		
1	<i>Salt Water.</i> 20 per cent. NaCl, at 91°-93° F. (Room air temperature, 74.5° F. dry-bulb, 63.5° F. wet-bulb)	1	45	12	33	—	—	122	1026	See note to experiment 6.	
		2	70	25	45	—	—				
		3	102	37	65	—	—	120	1026.		
		4	134	49	85	—	—				
		5	165	62	103	—	—				
		6	192	74	118	—	—				
2	<i>Ordinary Water</i> at 91°-93° F. (Room air tempera- ture, 75.8° F. dry-bulb, 64.1° F. wet-bulb)	1	19	12	7	—	—	195	1027*		
		2	30	25	5	—	—	161	1019		
		3	46	37	9	—	—	241	1014		
		4	63	49	14	—	—	137	1012		
		5	70	61	9	—	—	92	1014		
		6	80	73	7	—	—	70	1017		
3	<i>In Air, naked.</i> (Room air temperature, 72.8° F. dry-bulb, 57.5° F. wet- bulb)	1	60	13	47	—	—	166	1027		
		2	87	26	61	—	—				
		3	133	40	93	—	—	230	—		
		4	156	53	103	—	—				
		5	200	66	134	—	—				
		6	236	79	157	—	—				
4	<i>In Air, normally clothed.</i> (Room air temperature, 71.0° F. dry-bulb, 57.0° F. wet-bulb)	1	66	13	53	—	—	230	1025*		
		2	110	26	84	—	—				
		3	147	40	107	—	—	—	1028		
		4	174	53	121	—	—				
		5	215	66	149	—	—				
		6	253	79	174	—	—				

5	Ordinary Water at 91°-93° F. (Room air temperature, 68.0° F. dry-bulb, 63.5° F. wet-bulb)	1 2 3 4 5	13 22 37 46 50	12 23 35 46 58	1 -1 2 0 1	— — — — —	— — — — —	123 91 93 87 57	1023 1019 1019 1020 1026	Washed with cleansing powder before start and between each period.
6	Ordinary Water at 91°-93° F. (Room air temperature, 68.0° F. dry-bulb, 64.0° F. wet-bulb)	1 2 3 4 5 6	19 46 58 69 82 96	11 23 34 46 57 69	8 23 24 23 25 27	— — — — — —	— — — — — —	362 130 129 100 87 64	1025* 1011 1017 1016 1020 1022 1025	Washed with ether before start and after third hour. In this experiment the subject was, owing to the action of the ether, very cold on going into the bath; and this was probably the cause of the initial diuresis. A similar cause seems to have been present to some extent in other experiments.
7	Ordinary Water at 91°-93° F. (Room air temperature, 65.0° F. dry-bulb, 60.5° F. wet-bulb)	1 2 3 4 5 6	0 17 30 41 50 56	12 24 36 48 60 72	-12 -7 -6 -7 -10 -16	— — — — — —	— — — — — —	176 149 121 116 145 125	1025* 1021 1019 1020 1020 1017 1018	Washed with soft soap before start and between each period.
8	Salt Water. 11.5 per cent. NaCl, at 91°-93° F. (Room air temperature, 66.5° F. dry-bulb, 62.0° F. wet-bulb)	1 2 3 4 5 6	35 66 85 116 147 176	12 23 35 47 59 70	23 43 50 69 88 106	— — — — — —	— — — — — —	119 109 90 94 72 47	1024 1021 1023 1023 1021 1025	Washed with soft soap before start and between each period.
9	Ordinary Water at 99°-100° F. (Room air temperature, 76.7° F. dry-bulb, 63.5° F. wet-bulb)	1 2 3 4 5	— — — — 1080	— — — — 85	— — — — 995	98.6* 99.1 99.9 99.9 100.1 100.0	76* 94 91 92 96 96	48 34 18 16 13	1027* 1024 1029	Vomited at finish of experiment.

Table I—(continued).

Experi- ment No.	Kind of bath.	Period in hours.	Total measured loss in weight, grams.	Total loss in weight from lungs, grams.	Total loss in weight through skin, grams.	Average mouth tempera- ture per hour period, °F.	Average pulse rate per hour period.	Urine per hour period.		Remarks.
								Grams.	Specific gravity.	
10	<i>Salt Water</i> , 18 per cent. NaCl at 98°-100° F. (Room air temperature, 76.7° F. dry-bulb, 63.6° F. wet-bulb)	1	—	—	—	98.6	84	130	1015*	
		2	—	—	—	99.8	97	35	1016	
		3	—	—	—	99.9	98	27	1027	
		4	—	—	—	99.6	98	14	1031	
		5	2833	86	2747	99.8	97	17		
11	<i>Salt Water</i> , 1.1 per cent. NaCl at 90°-91° F. (Room air temperature, 76.2° F. dry-bulb, 62.7° F. wet-bulb)	1	—	—	—	98.2*	76*	163	1026*	
		2	—	—	—	98.2	71		1022	
		3	—	—	—	97.9	63			
		4	—	—	—	97.8	60	228	1015	
		5	67	62	5	97.7	55	64	1020	
12	<i>Salt Water</i> , 1.0 per cent. NaCl at 99°-101° F. (Room air temperature, 72.2° F. dry-bulb, 59.3° F. wet-bulb)	1	—	—	—	98.4*	78*	53	1027*	
		2	—	—	—	99.3	92	22	1030	
		3	—	—	—	100.4	95	23	1030	
		4	—	—	—	100.3	93	23		
		5	1413	93	1320	100.5	103	23		
						100.2	107	14		Vomited at finish of ex- periment.

* Prior to start of experiment.

per cent. NaCl). Osborne (1923) found that the concentration of salt solution had to be raised to 10 per cent. before the skin remained normal after one hour's immersion.

When the figures for the amount and specific gravity of urine excreted are examined, it is seen that in general the quantity is considerably greater, and specific gravity lower than normal, in ordinary water baths than in salt water. This result is exactly what might be expected from physiological considerations. The graph indicates that in the baths in pure water or isotonic salt solution about 29 c.c. less of water was passing off through the skin per hour than when the subject was sitting clothed in air, and 20 c.c. less than with a salt-water bath. We should therefore expect an extra 29 c.c. of pure water in the urine per hour, and this would account for the changes in the urine. The hot baths in experiments 9, 10 and 12 show a low excretion of urine, and a very large loss in weight, which is what might be expected since a considerable amount of sweating must have taken place—this being indicated by the great loss of weight through the skin by the subject. The subject was feeling considerably distressed at the conclusion of experiments 9 and 12, and vomited just after getting out of the bath. It will be seen, however, that in experiment 10, where the subject could lose water freely by osmosis, the water lost through the skin was about twice as much. The reason for this loss by osmosis being far greater than in the cooler bath will be given below.

In the ordinary water baths, it appears that any absorption of water through the skin occurred only to a slight extent. Practically no difference was shown with a 1.1 per cent. salt-water bath. In the stronger (11.5 and 20 per cent.) salt-water baths, the loss in weight showed that a considerable amount of water was passing through the skin into the salt water; and this would be the case if the skin were permeable to water, for the osmotic effect of the stronger salt-water baths must be much greater than the ordinary water baths, besides being opposite in direction.

In an investigation on some effects of hot baths, Bazett and J. B. S. Haldane (1921) observed a diuretic effect with baths of ordinary water just comfortably warm, and also found, like ourselves, that this was not accompanied by any gain in weight, and so could not be accounted for by passage of water inwards through the skin. It is not, however, passage of water inwards, but the prevention of passage of water outwards, that accounts for the diuretic effect observed when baths of pure water were used.

The osmotic passage of water through the skin must vary, other things being equal, with the depression of vapour pressure outside the skin as compared

with the vapour pressure of the blood, which is nearly equal to that of water. We should, therefore, expect that, since even beneath clothing the depression of vapour pressure in ordinary air* warmed to the skin temperature will be greater than in strong salt solution, the osmotic loss of water through the skin would be greater than in a bath of 20 per cent. salt solution, in which the depression of vapour pressure is only about 14·7 per cent. Experiment 4 showed that this was the case, and, as explained below, it was significant that the loss was greater than in experiment 3 with the subject naked. It has recently been shown by K. Mellanby (1932) that air beneath clothing is far from being saturated under ordinary resting conditions.

In order to obtain data as to osmotic loss from an unclothed subject with definitely known proportions of moisture in the air, and varying air-temperatures, we made a further series of experiments on another subject (H.W.L.) of nearly the same weight. As in successive experiments the air-temperature was raised until evident sweating began, it was necessary to distinguish osmotic loss from loss by sweating. This we did by determining, in the manner already described in previous papers, the amount of chlorine lost through the skin when this loss was appreciable, and counting this chlorine as indicating loss of water due to sweating, and the remaining loss of water as being osmotic.

It is evident that the loss counted as due to sweating would depend on the percentage of chlorine normally present in the sweat of the subject; and this was determined by raising the temperature and moisture of the air to a point where sweating was so marked that the whole skin was wet and dripping, so that no osmotic loss of water could occur. When this point was reached the chlorine percentage, as shown in Table II, was 0·124. In making the determination we avoided very profuse or long-continued sweating, since this tends to increase the chlorine percentage. The chlorine removed from the skin when the subject was washed down with distilled water after each experiment was determined as accurately as possible, and with plenty of wash-water. But it was found that if, without any exposure to warm air, the subject was washed down again in the same way, an appreciable, though extremely small and fairly constant amount of chlorine (about 0·025 gm.) was obtained, due probably to removal of loose epidermis during the second washing. This very small average amount was therefore deducted from the loss counted as due to sweating if the apparent loss was less than 0·05 gm.

In all the experiments there was a good ventilation (about 86 cubic feet per minute), maintained by a blowing fan, through the chamber, the dry and wet bulb temperatures being raised by heating and moistening the incoming air.

Table II.

Experiment No.	Average air temperature.		Vapour pressure of water at body temperature minus vapour pressure in the air, mm. of Hg.	Total weight lost in 2 hours, grams.	Loss in weight via lungs per hour, grams.	Net weight lost through skin per hour, grams.			Chlorine per hour, grams.	Chlorine per cent.	Remarks.
	Dry bulb, °F.	Wet bulb, °F.				By sweating.	By osmotic loss of water.	Total.			
I	75	60	38	100	17	—	—	33	—	—	Lightly clothed. At rest, sitting.
II	77	59	39	81	17	Nil	23	23	Nil	—	Naked. At rest, sitting.
III	76	61	38	205	29	23	50	73	0.029	0.040	Naked. Working on bicycle ergometer at rate of 1190 ft. lbs./min.
IV	76	65	34	315	30	29	98	127	0.036	0.028	Naked. Working on bicycle ergometer at rate of 2130 ft. lbs./min.
V	85	65.5	37	115	16	4	37	41	0.005	0.012	Naked. At rest, sitting.
VI	86.5	82.5	20	132	10	7	49	56	0.009	0.016	" " " " " "
VII	93.5	65	40	225	18	35	59	94	0.043	0.046	" " " " " "
VIII	93.5	66.5	38	240	16	31	73	104	0.038	0.037	" " " " " "
IX	91	70	34	540	32	113	125	238	0.140	0.059	Naked. Working on bicycle ergometer at rate of 2210 ft. lbs./min.
X	93	88	14	240	9	26	85	111	0.032	0.029	Additional air current produced by small electric fan.
XI	98	90	13	330	9	47	109	156	0.058	0.037	Naked. At rest, sitting.
XII	101.5	69.5	38	365	17	48	117	165	0.059	0.036	" " " " " "
XIII	114.5	90.5	18	(665)*	10	433	Nil	433	0.535	0.124	" " " " " "

* Duration of experiment 1½ hours only.

Table II shows in a striking manner the already well-known fact that with rise in air-temperature the loss of water through the skin increases rapidly. But it also shows the new point that during rest with air of ordinary dryness practically the whole of the loss was osmotic till the air-temperature reached 85° F., and most of it was osmotic till the air-temperature was well above body-temperature; also that the osmotic loss increased enormously with rise of air-temperature, short of the point which caused the skin to become wet.

With the air-temperature at 101°, for instance, the osmotic loss was 117 grams per hour. Evaporation of this amount of water from the skin would absorb about 68 kilogram-calories of heat per hour, or 1,630 per 24 hours, which corresponds to about the heat produced in the ordinary resting metabolism of a man; and it is evident that with the skin fairly warm osmotic loss of water through the skin plays an important part in regulation of body-temperature. As the air-temperature rises, loss of heat from the skin by conduction and radiation tends to diminish, in spite of increased skin-circulation; but this is compensated for by increased osmotic loss of water, until a point is reached where osmotic loss is supplemented by sweating which is at first insensible. With further rise of temperature the sweating increases until the whole skin becomes wet, so that osmotic loss is entirely prevented. As shown by the bath experiments, it could still, however, occur to a large extent if the sweat present visibly on the skin became very concentrated, as tends to occur in dry or rapidly moving air; and in experiment 10 of the bath experiments it appears that fully 200 grams per hour were lost by osmosis.

There is nothing surprising in the fact that osmotic loss of water increases so markedly with air-temperature. As the air-temperature rises the rate of skin-circulation certainly increases greatly, doubtless in consequence of the opening up of large numbers of capillaries which were previously closed. From the discoveries of Krogh we can infer with practical certainty that this must occur when the skin-circulation increases. The vapour-pressure of water in the blood passing through the skin increases, also, in consequence of the blood being warmer. Thus both increase in the number of open skin-capillaries and rise in vapour-pressure in the blood will contribute to the great increase in osmotic loss of water as the air-temperature rises, though the experiments described below on the rate of absorption of oxygen through the skin indicate that the former factor is by far the more important one. With the subject naked and the air-temperature 77° F., the osmotic loss was only 23 grams per hour, and was very little more than the loss by breathing. With a slightly lower temperature, but the subject clothed, the osmotic loss was increased to

33 grams, owing, clearly, to the skin being warmer. With increasing air-temperature up to 101.5° the osmotic loss increased to 117 grams, with a loss of 48 grams by sweating. At 114.5° the whole skin was wet and dripping, so that no osmotic loss could occur, and all the loss was by sweating, so that the water lost by sweating contains as much chlorine as normal sweat.

It will be seen that the effect of raising the dew-point of the air was not very marked before the dew-point of air saturated at body-temperature was approached. It would seem that raising the dew-point tends to raise the skin temperature, and that this increases the osmotic loss by nearly as much as raising the dew-point diminishes it. In order to give a measure of the extent to which, but for physiological regulation, raising the wet-bulb temperature would tend to diminish the rate of osmotic loss, we have added a column showing the difference between the vapour-pressure in the air and that in water at body-temperature.

In the investigation already referred to by Richardson, of the water given off by the skin of a subject with congenital absence of sweat-glands, it was found that even with the air so hot that the subject's body-temperature was rising rapidly in the absence of sweating, only about 33 grams per hour were given off. This is only about a fourth of what our experiments indicate as coming off independently of sweating with a normal subject. It would seem, therefore, that congenital absence of sweat-glands is accompanied by defect in skin-circulation. This defect must contribute greatly to the defective regulation of body-temperature with high air-temperatures or during muscular exertion. Richardson's experiments show with great clearness how very defective is the regulation.

Since raising the skin-temperature by raising the air-temperature has so great an influence in increasing the osmotic passage of moisture, we should expect to find that muscular exertion in cool air would have a similar effect, since it leads to increased circulation and rise in temperature of the skin. It will be seen from the third and fourth experiments in the table that this was the case, the osmotic loss of moisture being raised to 50 and, with nearly twice as much work, to 98 grams as compared with about 23 grams at the same air-temperature without muscular exertion. In a further experiment (No. 9) at a much higher temperature, and in a good air current, the osmotic loss rose to 125 grams, but the loss by sweating had increased to 113 grams, as compared with 29 grams in the previous experiment with work, so that the point was approaching at which osmotic loss would be extinguished by sweating. The skin was only definitely wet in places during this experiment, so that most of

the sweat was drying up. It is thus evident that osmotic passage of water plays an important part in the regulation of temperature during muscular work, as well as during rest.

It seems quite evident from this whole series of experiments that osmotic loss of water through the skin is under the same physiological control as the skin-circulation. The manner of this control is intelligible as being part of the maintenance of body-temperature; and cooling from osmotic loss of water adds greatly to the influence of varying skin-circulation in adjusting heat-loss from the body to either variations in surrounding temperature, or variations in heat-production within the body. When the skin becomes wet with sweat osmotic loss of water ceases; but it seems probable that in animals with no sweat-glands distributed generally over their skin, osmotic loss of water may play a more continuous part in regulating body-temperature than in man. In our experiments we have not needed to take into account the sweating from the palmar surfaces which, as Kuno (1930) has recently shown, occurs from emotional causes.

If we describe passage of water through the skin as osmotic it may be asked what the osmotic pressure capable of being given by the air is, since water is passing, or tending to pass, into it from the blood. The clear answer to this question is that the osmotic pressure given by the air is the pressure by which the existing air-pressure would require to be raised in order to make the vapour-pressure of the moisture in it equal to that of the blood in the skin, so that no moisture could be given off from the latter. To estimate this pressure, however, it is evident that not only would the skin have to be regarded as a rigid septum, but the temperature of the air would require to be equal to that of the skin. With the skin-temperature and air-temperature at body-temperature, and the pressure of aqueous vapour in the air the same as in experiment 2 (dry bulb 77° and wet bulb 59°), *i.e.*, 7.9 mm. of mercury, as compared with 47 mm. in air saturated at body-temperature, the pressure of the air would require to be raised to $47/7.9 \approx 6$ atmospheres to stop osmosis. The osmotic pressure of the air would therefore be $6 - 1 = 5$ atmospheres. The osmotic depression at the same temperature would be $1 - 7.9/47 = 0.832$ atmosphere. Only if the air was almost saturated at body-temperature would osmotic pressure and osmotic depression increase approximately parallel to one another, as is also the case with very dilute solutions.

Since the osmotic transference of water through the skin is so much influenced by air-temperature, we should expect that the transference of gases through the skin would be similarly influenced. Many experiments have been made on the

giving off of carbon dioxide by the skin; and Schierbeck (1893) in a series of experiments measured the amount of carbon dioxide given off at different temperatures. In these experiments the subject was immersed to the neck in a chamber through which was passed a large current of air, which could be heated to different temperatures. A definite fixed proportion of this air was passed through baryta water in Pettenkofer absorption tubes, so that the carbon dioxide given off could be measured. The general conclusion was that the amount given off was extremely small and practically constant until the temperature at which sweating began was reached. With further rise of air-temperature the amount was found to increase very rapidly; and the increase was attributed to the metabolism of the sweat-glands.

It did not, however, appear to us that increased metabolism of sweat-glands, or the very small amounts of CO_2 which are presumably present in the sweat itself, could account for the increased transfer of carbon dioxide. We wished, also, to determine the absorption of oxygen through the skin; so we made a new set of experiments. The method employed was very simple. An arm was placed in a glass cylinder (4 inches diameter), one end of which was fitted with a wide rubber band to fit round the arm, and the other end closed with a rubber bung. For air-sampling purposes, the bung was pierced by a length of glass tubing reaching half-way along the inside of the cylinder, the tubing projecting outside the cylinder being fitted with a short length of rubber tubing and a pinch-clip. A thermometer was placed in the cylinder during each experiment. Samples of the air in the cylinder were taken with evacuated tubes at the start and finish of each experiment, and in some cases samples were also taken half-way through. The volume of the cylinder not occupied by the arm, *i.e.*, the dead space, was measured with water and found to be 1670 c.c. The air samples were analysed with a Haldane air-analysis apparatus. As the volume of the dead space was known, the actual volumes of CO_2 and O_2 in the cylinder at the start and finish of an experiment could be calculated easily after the usual corrections had been made, thus giving the volumes of CO_2 produced and O_2 absorbed. These are given in Table III.

The area of skin surface exposed in the cylinder was measured experimentally. The arm was coated with molten paraffin wax, the latter removed when it had set, and finally flattened out after treatment in warm water. The areas of the pieces of wax were then measured with a planimeter, the total area being 0.100 sq. metre. The area of the whole body surface was calculated by means of the Du Bois formula, and amounted to 1.77 sq. metres. From these

Table III.—Passage of Gases through the Skin.

	Air temperature.		Temperature of air in tube, °F.	Volume of CO ₂ produced per hour c.c. at N.T.P.	CO ₂ produced by whole body surface per hour.		Volume of O ₂ absorbed per hour, c.c. at N.T.P.	O ₂ absorbed by whole body surface per hour.	
	Dry bulb, °F.	Wet bulb, °F.			c.c. at N.T.P.	Grams.		c.c. at N.T.P.	Grams.
(a)	49.0	—	73	1.20	21.2	0.042	Nil	Nil	Nil
(b)	62.4	49.7	75.5	2.02	35.8	0.071	0.46	8.1	0.012
(c)	75.0	—	80	2.46	43.5	0.086	0.62	11.0	0.016
(d)	70.0	—	87	4.41	78.1	0.154	1.21	21.4	0.031
(e)	73.0	—	87	4.71	83.4	0.165	2.43	43.0	0.061
(f)	96.2	62.9	95	9.15	162.0	0.320	3.24	57.4	0.082
(g)	94.6	90.3	98	14.30	253.1	0.500	4.27	75.6	0.108

data, the amounts of CO_2 produced and O_2 absorbed by the whole body surface per hour have been calculated, and are given in Table III.

These amounts can, however, only be regarded as approximate, since the skin no doubt possesses regional variations as regards CO_2 production and oxygen absorption.

It will be seen from Table III that, starting from an air-temperature of 49°F. , the amounts of CO_2 given off and O_2 absorbed increased continuously, and with rapidly increasing increments, as the air became warmer. This increase was independent of sweating, since, to judge from previous experiments, marked sweating would only be present in the last experiment, whereas the loss of CO_2 had increased to about eight times before this point was reached. We think that the apparent discrepancy between our results and those of Schierbeck is due to the unreliability, under the circumstances in which it was used when very little CO_2 was being given off, of the Pettenkofer method which he employed. At that time it was not generally known that baryta water acts appreciably on the glass absorption-tubes used in the Pettenkofer method, and to different extents in different tubes (1890).

The great increase in gaseous exchange with increase in air-temperature runs more or less parallel with the great increase in osmotic loss of moisture, and must, we think, be attributed to the same cause, namely, great increase in the circulation through the skin, accompanied by similar increase in the number or permeable capillaries. It does not seem probable that increase in the consumption of O_2 and production of CO_2 by the living tissues themselves with rise of temperature can have much influence, since the increased circulation will by itself bring extra O_2 and carry off the extra CO_2 . With increase in skin-temperature the pressure of CO_2 in the blood passing through the warmed capillaries will be increased, and this will favour the giving off of CO_2 ; but in the case of oxygen the raised oxygen pressure in the warmed blood will be unfavourable to the absorption of oxygen, and yet the oxygen absorption increases with air-temperature quite as markedly as the giving off of CO_2 . It thus appears that it is the increased circulation which is by far the most important factor; and this conclusion confirms that reached as regards the osmotic passage of water.

The fact that, volume for volume, three or four times as much carbon dioxide is given off by the skin as oxygen is absorbed seems intelligible when we take into account the fact that carbon dioxide is far more soluble in a watery liquid than oxygen.

The calculated volume of carbon dioxide given off per hour from the whole

of the skin is quite trivial, unless the skin is warm, as compared with the amount given off by the lungs. With the skin warmed, however, the volume rose to 253 c.c.—about 2 per cent. of what may be given off by the lungs during complete rest.

From the effects of warmth in increasing the penetration through the skin of both water and the two gases investigated, we may infer that the rate of penetration of other substances, such as oils, which are capable of penetrating will be similarly influenced by warmth. Clinical experience with remedies which can be administered through the skin seems to confirm this inference.

Although all our experiments on the relation of warmth to osmotic transfer through the skin have had to be made with the skin naked, they certainly apply also to conditions in which the skin is clothed, although with clothing the influence of warmth will appear at a much lower air-temperature. Provided, however, that moisture can pass freely through clothing, and thence to the atmosphere, osmotic loss of water will play just as important a physiological part with clothing on as with it off.

The main expenses of this investigation were covered by a grant from the Safety in Mines Research Board, to which our acknowledgments are due.

Conclusions.

(1) Of the moisture which is given off from the skin during rest under ordinary conditions of temperature, the whole, or a very large proportion, passes through by osmosis or diffusion. The osmotic loss was demonstrated through the different effects of baths in ordinary water, and strong salt-solution.

(2) The osmotic loss increases very rapidly as the skin-temperature rises; but with a sufficient rise a point is reached where the presence of liquid sweat (not greatly concentrated) over the whole skin interrupts the process completely.

(3) The osmotic loss is controlled physiologically in conjunction with the control of skin-circulation, and, when the skin is warm but not yet wet with sweat, plays a large part in the regulation of body-temperature with varying external temperatures and rates of heat-production within the body.

(4) The passage of gas through the skin runs more or less parallel with the osmotic passage of moisture.

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*Secretin.**

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The preparation of secretin has been described in a previous paper (1928). The method involved extraction of the duodenal mucosa with absolute alcohol and precipitation of the dissolved secretin by bile salts. It was based on two observations: (a) secretin exists in the duodenal mucosa in an active condition, and (b) the secretion of pancreatic juice is due primarily to the absorption of bile salts in the duodenum. The bile salt used in the precipitation of secretin was commercial sodium tauroglycocholate. Apparently many varieties of this commercial bile salt preparation exist depending on the type of bile from which it has been obtained. For this reason many experimenters have experienced difficulties in obtaining satisfactory yields of secretin by this method. The conditions under which pure taurocholic acid precipitates secretin from solution have been worked out, but since this bile acid is difficult to obtain the results are of small general interest. For these reasons the preparation of secretin by a method which requires no special reagents is described. The description is divided into three stages. At each of these stages the preparation may be left for some days without deterioration.

* A detailed bibliography is contained in the article on Secretin by E. U. Still, 'Physiol. Rev.,' vol. 11, p. 328 (1931).

Method.

(1) The duodenum and upper part of the jejunum (*i.e.*, 3 feet of gut from the pyloric sphincter) of the recently killed pig is used as the source of the secretin. The mucous membrane is scraped off within 3 hours of the death of the animal, ground up with sand, and extracted with a volume of ethyl alcohol equal to four times its weight. The mixture is well shaken, allowed to extract at room temperature for 30 minutes and then filtered. The clear filtrate, if kept in the dark in a cool place, retains its activity for several days. It may be observed that a solution of secretin containing bile pigment is destroyed rapidly by light.

(2) The filtrate is diluted with an equal volume of CaCl_2 0.1 N. The fats and soaps in the extract are precipitated, and, after standing for a few minutes, may be removed by a Chardin filter. The filtrate is distilled at a low temperature *in vacuo* to about one-quarter of its volume. As secretin is destroyed slowly during this distillation it is advisable to distil small quantities at a time. I have found that 500 c.c. is a convenient volume of filtrate to distil to about 125 c.c. The concentrated fluid, which, when cool, shows signs of precipitation, is acidified by the addition of acetic acid (30 c.c. of HA 1 per cent. to each litre of the distillate), and after a short time (15 minutes) the fluid is spun in a high-speed centrifuge. The pigmented gelatinous precipitate so obtained is extracted with successive small quantities of absolute alcohol and the insoluble residue is rejected. This concentrated solution of secretin in alcohol is added to four volumes of acetone. Crude secretin is precipitated which, after drying with acetone, may be kept for an indefinite period of time.

(3) This crude secretin is now extracted with HCl 0.05 N. The greater part of the precipitate dissolves, but the secretin remains undissolved in a highly concentrated form. This secretin product is deeply pigmented and from colorimetric tests it was found to contain 2 per cent. of bilirubin. To remove the bilirubin the product is dissolved in acid alcohol (*i.e.*, 100 c.c. absolute alcohol to which 0.3 c.c. HCl(C) has been added) and any undissolved residue rejected. The secretin is reprecipitated from solution by the addition of three volumes of acetone and may be dried by means of ether. A picrate may be made by dissolving this secretin in a small quantity of water and adding to it a solution of picric acid in water till the maximum precipitate is obtained. The picrate may be redissolved in a small quantity of 75 per cent. acid alcohol (75 per cent. alcohol containing HCl 3 M.) and the hydrochloride precipitated by 10 volumes of acetone.

The yield of secretin is about 10 mgm. from 500 grams of mucosa.

The Chemical and Physical Properties of Secretin.

Secretin is a white amorphous powder.

Solubility.—Secretin is soluble in water and dilute alkali, but is insoluble in dilute acid. It is sparingly soluble in ethyl alcohol but freely soluble in 80 per cent. alcohol. It is insoluble in ether, acetone, or chloroform.

Reaction.—Secretin dissolved in water has a reaction of p_H 5.5.

Dialysis.—Secretin dissolved in water does not pass through a collodion membrane. 2 mgm. of secretin dissolved in 5 c.c. of water were placed in a collodion sac which was suspended in 5 c.c. of water. After an interval of 5 hours 2 c.c. of the dialysate, injected intravenously into a cat, produced no secretion of pancreatic juice. A control injection of 0.02 mgm. of the secretin dissolved in water produced 40 drops of pancreatic juice in 20 minutes. The experiment was repeated in the same way except that 0.5 c.c. of cat's bile was added to the secretin solution. The secretin did not pass through the collodion membrane even under these conditions.

Precipitation of Neutral Salts.—Secretin dissolved in water is precipitated from solution by half saturation with ammonium sulphate.

Composition.—Secretin contains the elements carbon, hydrogen, nitrogen, oxygen and sulphur. It is free from phosphorus. The constancy of the physiological activities of a series of preparations made by the method described above appeared to justify the quantitative analysis of it. A micro-analysis of the secretin after drying to constant weight in a high vacuum over P_2O_5 gave the following results:—

	Per cent.		Per cent.
Carbon	49.09	Oxygen (by difference)	27.561
Hydrogen	6.887	Sulphur	1.5
Nitrogen	13.8	Ash	1.167

This analysis, considered in relation to its physical properties, suggests that secretin is a polypeptide. The colour reactions given by it and its rapid destruction by proteolytic enzymes support this assumption.

Colour Reactions.—The protein colour reactions were determined on a colourless 0.2 per cent. solution in water. The Biuret and Xanthoproteic reactions were positive. The following reactions were negative: Millon, Pauly, Ninhydrin and Glyoxylic.

Action of Trypsin.—Secretin is rapidly destroyed by trypsin. It is not acted upon by enterokinase or trypsinogen contained in freshly secreted pancreatic juice.

The Physiological Properties of Secretin.

The effects of secretin on the following physiological processes were determined: (a) the external secretion of the pancreas, (b) the internal secretion of the pancreas, (c) the secretion of bile, (d) the secretion of urine, and (e) the movement of the intestine and uterus.

(a) *The Secretion of Pancreatic Juice.*—The quantity of pancreatic juice secreted after the intravenous injection of a given quantity of secretin varies in different animals. Usually the injection of a given weight of secretin causes a much larger secretion in a well nourished than in a fasting cat. The weight of the cat appears to have little effect on the magnitude of the response except that a large pancreas secretes more juice than a small pancreas. Two experimental results are recorded to illustrate these statements.

Two cats, A and B, were anaesthetised with urethane (1.5 gm. per kilo of body weight administered subcutaneously). The pancreatic juice was collected after 1/40 mgm. of secretin had been injected into the femoral vein of each cat.

Rates of Secretion of Pancreatic Juice per 5 minutes.

Cat A (weight 2.5 k.).	Cat B (weight 3.8 k.).
16 drops	46 drops
15 "	23 "
10 "	9 "
4 "	3 "
2 "	1 "
Total secretion 47 drops in 25 minutes (2.3 c.c.).	Total secretion 82 drops in 25 minutes (4.1 c.c.).

The subcutaneous injection of relatively massive quantities of secretin produces a slow and prolonged secretion of pancreatic juice after a latent period of about 15 minutes. This long latent period is in marked contrast to the latent period usually observed after the intravenous injection of secretin (about 50 seconds). It precludes the possibility of some of the subcutaneously injected secretin having entered the blood stream direct.

The number of drops of pancreatic juice secreted in successive intervals of 15 minutes after the subcutaneous injection of 2 mgm. secretin dissolved in 10 c.c. H₂O into a cat (3.75 k.) anaesthetised with urethane were: 0, 2, 5, 10, 13, 7, 3. The total pancreatic juice secreted over a period of 2 hours was

2 c.c.—a quantity which the intravenous injection of 1/40 mgm. of secretin produced in 20 minutes.

The pancreas shows no sign of fatigue, estimated by the volume of juice secreted, after the intravenous injection of large quantities of secretin extending over a period of 6 hours—in fact the tendency is for the amount of juice secreted after the injection of a definite weight of secretin to increase slightly with each succeeding injection. The successive fractions of juice become progressively poorer in enzymes until the final secretion contains only small quantities of enzymes in a dilute solution of sodium bicarbonate.

(b) *The Internal Secretion of the Pancreas.*—It is now agreed that the intravenous injection of small quantities of secretin has no direct influence on the blood sugar. But the question arises whether the injection of a massive quantity of secretin which is capable of producing a volume of pancreatic juice comparable to that required for the digestion of a large meal influences the blood sugar in an indirect way. The question was investigated in the course of an experiment on a rabbit, deprived of food for 24 hours, to determine whether large doses of secretin produced toxic effects in the unanæsthetised animal.

1 mgm. of secretin dissolved in 5 c.c. salt solution was injected into the marginal vein of the ear of a rabbit (4·5 k.). The injection was repeated 2·5 hours later. The blood sugar was determined before and at half-hourly intervals after the injections.

Time.	Percentage of blood sugar.
Before injection of 1·0 mgm. secretin	0·100
0·5 hours after injection	0·111
1·0 „ „	0·100
1·5 „ „	0·097
2·0 „ „	0·104
Second injection of 1·0 mgm. secretin—	
1 hour later	0·113

The quantity of secretin injected into the rabbit was such that if divided into 1/40 mgm. doses and injected into a cat about 150 c.c. of pancreatic juice would have been secreted. The results conclusively prove that secretin has no influence on the blood sugar either by direct action on the pancreas or indirectly by causing a large secretion of pancreatic juice. The rabbit showed

no toxic symptoms during the experiment indicating the innocuous nature of the secretin preparation.

The Secretion of Bile.—It is evident that the hypothesis previously put forward—that the secretion of bile by secretin is secondarily due to the production of metabolites by the pancreas—is untenable since Still (1931) has shown that the effect still occurs in a depancreatized dog. It was of interest to determine therefore whether this preparation of secretin which is free from bile salts and bile pigments augmented the continuous secretion of bile.

A cannula was tied into the gall bladder of a cat anaesthetised with urethane, the common bile duct having been ligated. The viscid bile in the biliary passages was washed out with salt solution and the bile collected until a constant rate of secretion was established. The effects of the intravenous injections of secretin on the flow of bile and pancreatic juice were then determined. The following results were obtained :—

Secretin injected.	Pancreatic juice.	Increase of pancreatic secretion.	Bile.	Increase of bile secretion.
mgm.	drops	drops	drops	drops
0	0	—	18	—
1/40	32	32	23	5
1/20	68	68	27	9

It is evident from these figures that secretin has only a small effect on the continuous secretion of bile, compared with the large effect on the secretion of pancreatic juice, but this effect is approximately proportional to the quantity of secretin injected.

The intravenous injection of secretin simulates in a rough way only the natural absorption of secretin from the alimentary canal during the digestion of a meal in the intact animal. Possibly therefore the augmented secretion of bile under these conditions involves a mechanism to protect the animal against the presence of a large excess of secretin in the blood. To investigate this hypothesis 0.1 mgm. of secretin was injected intravenously into an anaesthetised cat and the bile secreted (1 c.c.) was collected during the following hour. This bile (1 c.c.) was now injected intravenously into the cat. As a result 13 drops of pancreatic juice were secreted during the succeeding 15 minutes. It is evident therefore that secretin is excreted in the bile after the intravenous injection of large doses. Possibly therefore the mechanism indicated above explains this slight chologogue action of secretin. The whole problem of

biliary secretion must be investigated before any definite conclusion can be reached.

(d) *The Secretion of Urine.*—The intravenous injection of secretin diminishes the secretion of urine, a result which may be secondary to the large secretion of pancreatic juice.

Cannulæ were tied into the pancreatic duct and urinary bladder of a cat anæsthetised with urethane. Successive quantities of secretin contained in 0·5 c.c. of water were injected into the femoral vein and the quantities of pancreatic juice and urine produced during periods of 1 hour were noted.

Time.	Secretin injected.	Pancreatic juice.	Urine.
	mgm.	drops	c.c.
1-2 p.m.	0·5	40	3·0
2-3 p.m.	0·025	30	0·5
4-4 p.m.	0·06	60	0·2
4-5 p.m.	0·075	70	0·2

At the end of the second hour the secretion of urine had practically ceased.

After the injection of large quantities of secretin the urine secreted may contain appreciable quantities of secretin. The urine secreted by a cat during a period of time in which several injections of secretin were made into the femoral vein was collected. The intravenous injection of 5 c.c. of this urine stimulated the pancreas to secrete 25 drops of juice in the succeeding 21 minutes. It is evident therefore that the kidneys in addition to the liver protect the animal against the presence of excessive quantities of secretin in the blood stream.

(e) *The Movements of the Intestine and Uterus.*—The absence of the Pauly reaction indicates that the secretin molecule does not contain the iminazol ring. It was of interest therefore to determine the action of this preparation of secretin on the smooth muscle of the intestine and uterus.

The action on a strip of intestinal muscle (rabbit) suspended in oxygenated tyrode solution was the same as that previously described—a concentration of secretin 1:300,000 increased the tone of the muscle to a considerable degree. Secretin has a much more powerful action than histamine on the contraction of the intestinal muscle of the rabbit. Comparative experiments indicate that the relative activity of secretin to histamine, in this respect, is about 10 to 1.

In the case of the guinea-pig's uterus entirely different relations are observed. This preparation suspended in salt solution gives a large contraction in a

concentration of histamine as low as 1 : 15,000,000. On the other hand a concentration of secretin as high as 1 : 30,000 has no effect on the tone or contractions of the uterus. The experiments indicate that the action of secretin on smooth muscle is limited to the musculature of the intestine.

The expenses of this work were defrayed by a grant from the Government Grant Committee of the Royal Society.

Summary.

(1) Secretin may be prepared from the duodenal mucous membrane by (a) extraction with absolute alcohol, (b) precipitation by dilute acid, (c) resolution in acid alcohol and precipitation by acetone.

(2) The yield of secretin is about 10 mgm. from 500 gm. of mucous membrane.

(3) The intravenous injection of 0.02 mgm. of secretin causes the secretion of about 40 drops of pancreatic juice in a cat.

(4) Secretin is a white amorphous powder, soluble in water and alkali but insoluble in acid. It is insoluble in acetone, ether and chloroform.

(5) Secretin has the percentage composition of a protein. It is rapidly destroyed by trypsin. Aqueous solutions do not dialyse through a collodion membrane.

(6) The physiological actions of secretin are : (a) the production of a large volume of pancreatic juice, (b) the contraction of intestinal muscle, and (c) the secretion of a small quality of bile.

(7) Secretin is excreted in the bile and urine after the injection of comparatively large quantities into the blood.

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*The Effect of Population Density upon Rate of Reproduction with
Special Reference to Insects.*

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I. *Introduction.*

As the result of a previous investigation, involving the analysis of environmental factors controlling population increase in nature, it became apparent to the writer that population density constituted an environmental factor which had been comparatively neglected, doubtless on account of its obscurity, but whose effects were nevertheless of greater significance than generally realised. It seemed desirable, therefore, that this relatively obscure phenomenon should be investigated, and the writer commenced work at the Laboratory of General Physiology, Harvard University, U.S.A.

It has long been known that crowding of animals produces definite and peculiar effects on the various vital processes, and even the morphology of the individual. For instance, as far back as 1854, Hogg produced evidence to show that a snail kept in a small cell would only grow to such a size as would enable it to move about freely. Later, Semper (1874) came to the conclusion that there was a relationship between volume and the ultimate size of the individual. As regards the effect of crowding upon reproductive rate the first observation appears to have been made by Balbiani (1860), who reported that *Paramecium* must be placed in not less than 2–3 c.c. of medium to bring about maximum productivity. Again, Farr (1843) showed that there existed a definite relationship between the density of the human population and the death-rate, and anticipated the trend of modern biology to the extent of elaborating a mathematical formula which conformed to his findings.

There is, however, another aspect of the situation, Robertson (1921) having found that a certain degree of crowding resulted in a stimulation of the multiplication rate of two infusorians, *Enchelys* and *Colpodium*. This phenomenon was designated by Robertson "allelolysis," the accelerated multiplication being attributed to the diffusion of some stimulating agent from the cells into the culture medium. Numerous subsequent workers have, however, failed to confirm Robertson's findings. These include Cutler and Crump (1923), Myers (1927), and Jahn (1929). Petersen (1929), using *Paramecium caudatum*

in her experiments, could produce positive and negative effects at will by manipulating the volume relationships.

The above brief summary gives some idea of the confused situation which prevails with regard to the effects of crowding upon the individual, and it is not intended to present here a complete review of the literature on the subject, as many of the experiments are so designed as to render impossible any definite incontrovertible conclusion. This is largely due to the practical difficulties involved in the stabilisation of a fluid medium containing several organisms, the three main uncontrollable factors being (1) the bacterial population in the culture—where their presence is essential as food supply, *e.g.*, infusorians, entomostracans, etc.; (2) the rapid diffusion of excretory products throughout a fluid medium; and (3) the possibility of toxic substances in small amounts dissolved from the glass containers. Hence to obtain convincing evidence of a "density effect," and, if possible, to express it in quantitative terms, it is essential to utilise a stable culture medium, and preferably, one which can be renewed as often as seems desirable. Obviously, a solid medium from which the organisms can be easily removed is the one which most closely approximates to the above requirements. In almost all other instances, what really takes place is a multiple effect consisting of the true density effect in itself plus that of any one or all three of the forementioned uncontrollable factors.

II. Quantitative Evidence from Recent Experiments.

Drosophila melanogaster.—The first convincing quantitative evidence of a true density effect was produced by Pearl and Parker (1922), Pearl and Surface (1909) having previously suggested its possibility, at the same time producing some evidence to substantiate their claims. The former collaborators made a valuable contribution to the problem by their analysis of the effect of population density upon the specific or net reproductive rate of *Drosophila*. This variable is measured by the average number of adult flies produced per mated female per day over a specified period of time, being in reality a measure of what is now generally termed the effective reproductive rate.

Pearl and Parker (*loc. cit.*) found that the number of flies (adult offspring only were counted) produced per natal pair fell continuously as the number of pairs per half-pint bottle was increased from 1 to 50, other conditions being standardised. The great definiteness with which this density factor apparently operates was convincingly demonstrated, at least over the comparatively short range tested out.

Tribolium confusum.—Further evidence is obtainable from the population studies of Chapman (1928) on the "Confused Flour Beetle" (*Tribolium confusum*), the experimental results of which have been analysed by the writer with the object of throwing more light on the problem in hand. This work of Chapman is extremely fortunate in many respects, the most important being that the experiments were not undertaken for the purpose of demonstrating a density effect (whose existence Chapman either dismisses or ignores) although the material and conduct of the experiments render them ideal for the purpose. The environment, in this instance, consisted of whole wheat flour, renewed at intervals of about 15 days, the initial population density being adjusted at will by maintaining constant the amount of flour and varying the number of beetles added to the floury medium, or *vice versa*. Both methods were used by Chapman in his population studies, which were designed to demonstrate that irrespective of the initial population density a point of equilibrium is eventually attained after which the population remains relatively constant, and that the time taken to reach this equilibrium population varies directly with the quantity of flour available, *i.e.*, the size of the environment. Chapman concludes that equilibrium is attained when the biotic potential is equalled by the environmental resistance, and that the lack of population increase is not due to absence of eggs or their infertility, but on account of the eating of eggs, pupæ and, to a lesser extent, the larvæ, by the adult beetles. It is perfectly obvious, however, that this explanation of the stationary character of the population is only partially correct, there being another important factor operating to bring about this state of equilibrium, as can be shown from the following analysis of Chapman's data.

We may take for examination the data presented in Table III of his paper, in which he records the population, at various intervals of time, where 2, 4, 8, 16, 32 and 64 beetles respectively, are placed in 32 grams of flour, making an initial population density, in geometric series, of 0.062 0.125 . . . 2.0 beetles per gram of flour. By the 25th day of the experiments no new adults had yet made their appearance, as the complete life-cycle takes a little longer under the temperature humidity conditions which obtained. Hence, by removing the initial adult population from the total population on the 11th and 25th days respectively, it is possible to determine the effect of the different initial densities upon the reproductive rate, assuming, as Chapman found, that almost all the eggs are fertile. This information, from Table III of his paper, is shown graphically in fig. 1, and is given in Table I.

Now, it will be observed from fig. 1 that there is a striking relationship |

Table I.—Recalculated from Chapman's Data.

Number of beetles :	2.	4.	8.	16.	32.	64.
Beetles per gram	0.062	0.125	0.25	0.5	1.0	2.0
Population in 11 days	37	176	288	512	864	1344
Population in 25 days	80	384	608	640	868	1344
Progeny per beetle in 11 days	17.5	43.0	35.0	31.0	26.0	20.0
Progeny per beetle in 25 days	39.0	95.0	75.0	39.0	26.1	20.0
Progeny per beetle per day (11 days)	1.59	3.90	3.18	2.82	2.36	1.82
Progeny per beetle per day (25 days)	1.56	3.80	3.0	1.56	1.04	0.80

Original environment in each experiment was 32 gm. of flour.

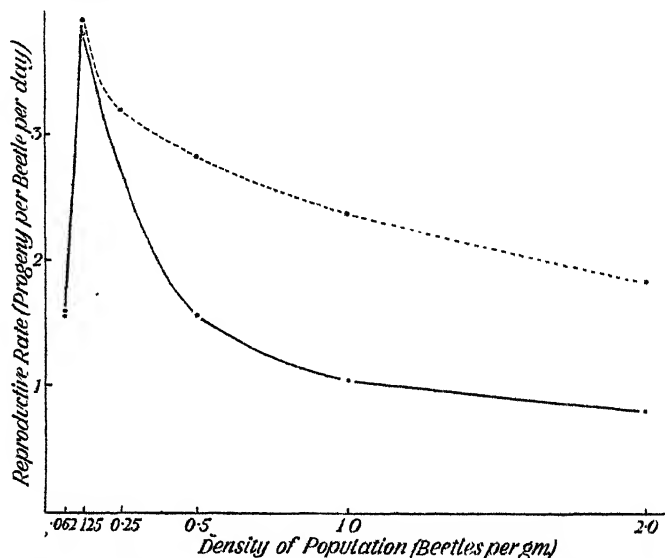


FIG. 1.—Density of population (*T. confusum*) in beetles per gram.

--- = over an 11-day period.

— = over a 25-day period.

between population density and rate of reproduction, the significance of the illustration being enhanced by the fact that the original experiment was not designed to demonstrate this phenomenon, although ideal for the purpose. In addition it is shown that there is an optimum density for rate of reproduction.

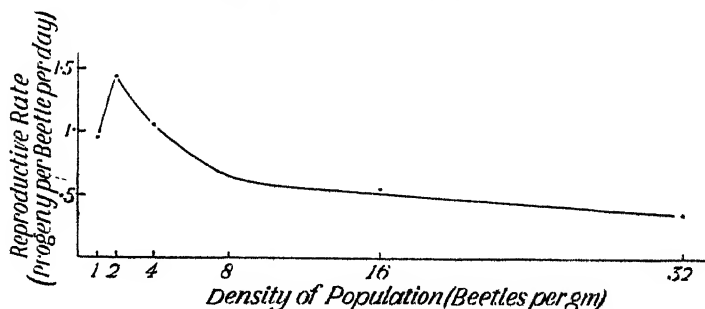
As indicated in fig. 1, the initial population density of 1 beetle per 8 gm. of flour brings about the maximum rate of reproduction. Taking the reproductive rate at the initial density of 0.125 beetles per gram, over the 25-day period, as 100 per cent., the rate at twice this density is 79 per cent., at eight times it is 27 per cent., and at 16 times it is 21 per cent. The very marked optimum density relation, combined with the differential effect exhibited by the curve of organic response above the optimum density, are strong

arguments against any possibility of explaining the phenomena presented in fig. 1 as being entirely the result of a purely mechanical effect, such as eating of the eggs or interference with copulation, when we would obtain a response directly proportional to the degree of crowding, or as the result of carbon dioxide accumulation. However, in order to obtain further evidence in regard to the above points the following additional experiments with *T. confusum* were undertaken.

In these experiments it is essential that the floury medium be reduced to the smallest possible dimensions consistent with the maintenance of a beetle population sufficiently large to ensure statistically reliable results. The object is to minimise the chances of carbon dioxide accumulation within the medium, and to facilitate the somewhat laborious process of removing, and counting, the eggs liberated by the beetles. The experiments were arranged in the manner indicated in Table II and were run at a temperature of 30° C. and 90 per cent. relative humidity, the medium being renewed at intervals of 48 hours.

Table II.—Beetles of Various Ages.

Number of beetles :	16.	16.	16.	8.	16.	32.
Flour in grams...	16	8	4	1	1	1
Beetles per gram	1	2	4	8	16	32
Eggs end of 2nd day ..	15	24	10	2	29	17
Eggs end of 4th day ..	27	51	31	12	14	24
Eggs end of 7th day ..	63	79	78	19	22	36
Eggs end of 8th day ..	15	29	14	3	4	12
Total eggs	120	183	133	36	69	89
Progeny per beetle per day ..	0.94	1.43	1.04	0.56	0.54	0.35

FIG. 2.—Density of population (*T. confusum*) in beetles per gram.

The results of those experiments are presented graphically in fig. 2 and confirm the evidence derived from the analysis of Chapman's data. The operation of the same biological law is clearly indicated in that, despite a changed optimum

density, the reproductive rate at any given density bears a constant relationship to the optimum density, *e.g.*, from Chapman's data it appears that the reproductive rate at twice the optimum density is 0.79 of the reproductive rate at the latter, and in the writer's experiments the ratio is 0.73. Again, at four times the optimum the figure is 0.41 in Chapman's experiments, while the writer's results give a ratio of 0.39. The relationship is extraordinarily constant, notwithstanding a slightly different rate of reproduction and a changed optimum density. The latter is an important consideration, and affords a good illustration of the fact that the optimum density for rate of reproduction is not absolute, but can be shifted as the result of changed environmental conditions, which may be physical or organic in character.

Additional experiments indicate that, in this instance at least, the changed optimum density was not due so much to a difference in physical environmental factors (although perhaps partly so) as to the evolution of a strain of beetles which were not only better adapted to withstand crowded conditions but actually found the conditions of life under such circumstances more agreeable, as evidenced by a slightly higher rate of reproduction in the highest densities as well as a shifting of the optimum to a more intense degree of crowding than obtaining in Chapman's experiments. The stock culture, from which the beetles used were taken directly, was maintained continually for 6 months under conditions of intense crowding and at a temperature varying between 21° and 25° C., thereby ensuring insects suitable for this type of biological experimentation, in that the making of numerous physical contacts in a somewhat vitiated atmosphere were common factors to their "normal" environment. Also, it should be noted, that as the experimental insects were taken at random from the stock culture, beetles of all ages from 1 to 5 months were included. This mixed population therefore had a lower reproductive rate than that of insects recently emerged from pupation, although higher than that of similar beetles previously maintained under less crowded conditions, on account of the release from the temporary inhibiting effect of crowding upon the reproductive rate of the younger members of the population.

In this connection it is interesting to observe that in those experiments with *Tribolium* the number of eggs produced per beetle per day almost invariably increased up to the 6th or 7th day, and thereafter decreased until a comparatively stable rate was attained. Of greater significance, however, is the fact that in this prestabilisation period the reproductive rate at the respective densities exhibits a differential effect in time, namely, the temporary stimulus diminishes in its rate of appearance from a maximum at the optimum density

to nil in the higher densities. With the latter there is a continual decrease in reproductive rate before the attainment of stabilisation. This phenomenon seems to indicate, so far as the writer can ascertain, a temporary phase of disharmony between organism and environment, during which there takes place (in some peculiar manner—probably psychological) a “sensitisation” of the organism, as indicated by a restoration of equilibrium between the reproductive rate and the environmental resistance. This phase may be aptly described as a “period of sensitisation to environment,” but is not to be confused with the so-called “latent periods” of various physiological phenomena.

Calandra granaria.—To obtain further knowledge regarding the possible mode of operation of the density factor and to confirm the operation of the density effect in other species, additional experiments were performed using for the purpose the grain weevil, *Calandra granaria*. This insect has certain advantages in that it differs from *Tribolium* in the deposition of the eggs, which, instead of being distributed at random in the floury medium, are inserted into the grains of the particular cereal being used. This considerably reduces the possibility of mechanical injury to the eggs by the movements of the weevils themselves, while the interstices between the grains render the effect of carbon dioxide accumulation even less probable than in a frequently changed environment of flour. To be strictly correct, the environmental unit of space, so far as oviposition is concerned in *C. granaria*, is the number of available grains per female weevil rather than the total volume per weevil.

A number of experimental environments, arranged so that the initial population of weevils per gram of wheat was in geometric series, were set up as follows: 0.25, 0.5, 1.0, 2.0 32.0 weevils per gram of wheat. The experiments were conducted at a temperature of $25^{\circ}\text{C.} \pm 1^{\circ}$, and a relative humidity of 90 per cent., as those conditions were found to be highly favourable for rapid reproduction in *Calandra*. In all the *Calandra* experiments half of the weevils were males and the other half females so far as could be determined from the length of the rostrum, and judging from the uniformity in the results obtained this mode of differentiating the sexes was quite successful, although, of course, only the very short and very long rostrated weevils were used. Since, in the act of oviposition, the female weevil moves about amongst the grains, inserting a single egg inside each and covering the aperture with a glutinous secretion, it is impracticable to count the actual number of eggs deposited. Hence, instead of the latter procedure, it was decided to count the number of adult weevils emerging within a known period of time. The

stock culture, from which the weevils utilised in the actual experiments were obtained, was kept for almost a year under highly favourable conditions of temperature and humidity for rapid reproduction, as well as being subjected to somewhat intense crowding, thereby ensuring a highly inbred strain of weevils on account of the selective elimination which occurs under these circumstances. Such strains are extremely desirable in biological experimentation bearing upon the analysis of population growth.

The results of the density experiments with *Calandra* are given in Table III and fig. 3, and it will be seen that the two variables, population density and

Table III.

Weight of grain (gram)	16	16	16	16	16	8	8	8
Initial number of weevils.	4	8	16	32	64	64	128	256
Weevils per gram	0.25	0.50	1	2	4	8	16	32
Number of grains per weevil	100	50	25	12.5	6.25	3.12	1.56	0.78
Population in 64 days	69	95	138	167	192	77	51	29
Progeny per weevil	17.2	11.8	8.6	5.2	3.0	1.2	0.4	0.1

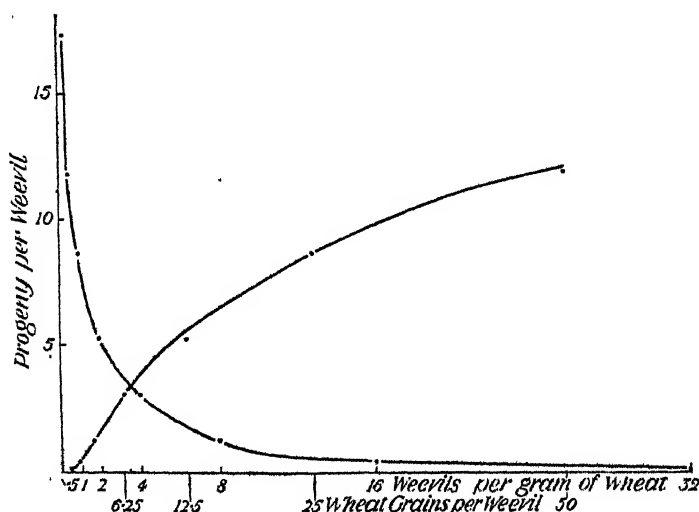


FIG. 3.—The scale on the ordinate is common to both curves, the upper one on the abscissa refers to the hyperbola.

progeny per weevil, exhibit a hyperbolic relationship. Further, if the same data is presented so as to demonstrate the relation between progeny per weevil and the average number of grains available per weevil, it is equally evident that the relationship of those two variables is not a rectilinear one, despite what might with some justification have been anticipated.

In fact, the curve obtained by plotting the last named variables brings out some very interesting points. Firstly, the female weevil never utilises the total number of grains at her disposal, for purposes of oviposition, no matter how hard pressed she may be for space ; and secondly, although the progeny per female weevil decreases as the number of grains at her disposal decreases, she utilises a greater percentage of the total available number, that is, the less space available the more efficiently does the female weevil utilise it, so far as oviposition is concerned, at least up to a certain degree of crowding. Thus, the highest average number of progeny per mated female weevil obtained under the temperature and humidity condition of these experiments, was 34.4, although the average number of grains at her disposal was 200, which means that of the total available space only approximately 17 per cent. was used. But, when the number of grains is reduced to 50, approximately 34 per cent. of them are used, and when still further reduced to 12.5 the percentage used is 48. Beyond this degree of crowding, however, a change takes place which results in an ever decreasing percentage of the total space being utilised for oviposition, until eventually, when the intensity of crowding is such that there is only one grain for every four female weevils, and the population consists of equal numbers of both sexes, the insects either cannot or will not oviposit at all. This comparatively sudden change in behaviour is undoubtedly due to the appearance of some other factor which does not operate in the lower densities, and is presumably physical in character, such as undue interference in the act of oviposition resulting from repeated physical contacts.

Perhaps the most significant fact resulting from this analysis of the "space factor" is that the female *Calandra* will not lay the maximum number of eggs produced under the conditions of these experiments until the number of grains available are at least eight times that actually utilised. This does not occur until there are 400 grains to every female weevil, when the number of males and females in the population are equal. Even at the "optimum" in regard to space utilisation for purposes of oviposition, nothing would induce the female weevil to utilise more than approximately 50 per cent. of the total available number of grains. Since this occurs when the average number of grains to each female weevil is 12.5, it follows that the densities productive of maximum oviposition and maximum space utilisation for the latter purpose, are by no means synonymous. Moreover, further experiments with *Calandra* indicate that if the number of wheat grains be increased from 400 per female weevil to 800, or even 600, there follows a reduction in the number of progeny, which

means there is an optimum degree of crowding in regard to number of eggs oviposited. This substantiates the evidence regarding an optimum density for reproduction already derived from our analysis of the *Tribolium* experiments.

Inter-specific Effects.—Having thus presented and examined, in some detail, the evidence regarding a true density effect, it was decided to try to obtain further information regarding its mode of operation by means of a juggling with numbers and space through the utilisation of “dummy grains” (glass beads) and the addition of another species to the environment. In this way it was possible to maintain the total available space for oviposition constant, while varying the total volume, and *vice versa*, remembering the distinction between the two. Considering first the question of interspecific effects upon reproductive rate, the following preliminary results were obtained, using the Angoumois grain moth (*Sitotroga cerealella*) and the Grain Weevil (*Calandra granaria*) as the interacting species. Both these insects breed with extraordinary rapidity in wheat, when temperature and humidity conditions are favourable, and these were maintained, as before, at $25^{\circ}\text{C.} \pm 1^{\circ}$, with a relative humidity of 90 per cent.

A series of experimental environments were set up, in which the ratio of weevils to moths in the initial population was varied, while the number of grains of wheat was maintained constant at 1000. Now, in a mixed population of 10 weevils and 20 moths, the progeny per moth was 11.6, in so far as can be judged from the number of emerging adults. However, when the initial population consists of 10 weevils and 40 moths (equal numbers of both sexes) the progeny per moth is only 8.7, and an initial population consisting of 40 weevils and 10 moths still further reduces the progeny per moth to 5.4. Since an initial population consisting of 10 moths and no weevils, in the same space, results in a progeny number of 18 per moth, it is apparent that the addition of the weevils to the environment brings about a profound reduction in the moth population. That the effect is not simply a mechanical one, such as destruction of the moth eggs by the weevils, is shown from further experiments in which the initial moth population was maintained constant while increasing the number of weevils, the reduction in the moth population being by no means proportional to the concentration of *Calandra*. A better insight into the matter is, however, obtained by observing the effect of the moths upon the weevil population, since the eggs of *Calandra* are better protected from mechanical injury by being inserted inside the grains of wheat, instead of being strewn about the surface. Since the reproductive rate at different

densities of this inbred strain of weevils has already been determined, the deviation of the actual number of progeny from the expected figure, due to the addition of the moths to the environment, can now be calculated.

Table IV.

(a) Weevil; (b) Moth.	0		10		10		20		10		40		40		10	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Total progeny (one generation)	0	180	131	233	89	349	340	54								
Progeny per individual	0	18.0	13.1	11.6	8.9	8.7	8.5	5.4								
Progeny per weevil at density = (a + b)		—		10.0		7.4		7.4								

The original environment consisted of 1000 grains of wheat.

Thus, in these experiments, Table IV, when the initial mixed population is 10 weevils and 20 moths, the progeny per weevil is 13.1, but we already know that the progeny per weevil at a density of 100 grains of wheat per weevil is 17.2, so that the presence of the moths caused a reduction of 24 per cent. The probable error in the *Calandra* results is not more than 4 per cent. Again, when the initial population consists of 10 weevils and 40 moths, the progeny per weevil is 8.9, i.e., a reduction of 8.3 per cent. from the expected figure. But, the addition of 10 moths to a population of 40 weevils in 1000 grains of wheat is not sufficient to cause any significant reduction from the expected figure if no moths were present, although the effect of the weevil upon the moth population at this density is very considerable. It will be observed from the first two mentioned figures that the reduction in the progeny per weevil is apparently inversely proportional to the numbers of moths, but this is accidental in that the relationship appears to hold over this short range and relative concentration of weevils and moths. We must now turn to a brief consideration of the data obtained from the experiments concerning the partial substitution of wheat grains by glass beads, as the results are of considerable interest.

Use of "Dummy" Grains.—In these experiments which were arranged as in the earlier series when determining the effect of density upon reproductive rate of *Calandra*, an equal number of glass beads were added to the wheat grains, thus making the number of grains per weevil the same as before, but in double the volume. (The glass beads were smooth and round, with a hole through the centre, and of approximately the same size as a grain of wheat.) This arrangement rendered possible the separation of available oviposition space and total volume, by which means it was intended to deceive the weevils

of the actual oviposition space at their disposal. It seemed *a priori* impossible to decide whether doubling the volume of the same number of grains by the substitution of "dummies" would result in an increased number of eggs being deposited as the result of more volume per weevil, or whether it would result in a decreased number on account of the increased space to be traversed; hence it was much to the writer's astonishment to find on analysing the results that the weevils behaved almost exactly as before, *i.e.*, as if the beads were to all intents non-existent.

For instance, when the number of actual wheat grains per weevil is 100, and the total volume is doubled through adding an equal number of "dummies," the progeny per weevil is found to be 16.5, and it will be recalled that the figure obtained previously for this density was 17.2, *i.e.*, a non-significant deviation. Again, when the number of grains per weevil is 6.25 and the total volume doubled by addition of an equal number of "dummies," *vide* Table V, the

Table V.

Number of weevils	64	32	8	4
Grains per weevil	6.25	12.5	50	100
Progeny per weevil	2.9	5.4	9.1	16.5

Original environment of 400 grains + 400 "dummies."

progeny per weevil is 2.9 while the figure obtained previously at this density when no beads were used, was 3.0. While the "dummy" grains therefore failed to bring about any significant alteration in the reproductive rate of the weevils within the above range of densities at any rate, there is some evidence which suggests that the above mentioned possibilities of the effect of the "dummies" do operate outside the above tested range, and that at very high densities the increased space induces slightly increased oviposition, whereas the substitution of "dummies" brings about a slight reduction at very low densities. Further confirmatory evidence, especially in view of the relation to insect parasites, is, however, essential before a definite conclusion can be reached.

III. Interpretation and General Biological Significance of the Results.

Effective Factor at Densities below the "Optimum" for Reproductive Rate.—It is now necessary to discuss the explanation and general biological significance of the density effect on the reproductive rate of the individual. In the first place, the possibility that the accumulation of carbon dioxide or other excretory product, might be responsible for the phenomena seems untenable in view of

the results of the experiments, which were designed to obviate this. Nor is the effect simply due to a difference in death rate, since it tended to be slightly higher in the lower densities, on account of the smaller number of beetles employed. Then there is the possibility of the production of a larger number of non-fertile eggs in the higher densities : by allowing the eggs to develop and counting the emerged larvæ the writer found this to obtain in the *Tribolium* experiments. However, the percentage increase in the number of eggs which failed to produce larvæ was extremely small in comparison with the drop in the number of eggs deposited.

The frequency and chances of interruption of copulation in the various densities must also be considered. This is undoubtedly an important factor, since it was found from continual observation of the insects' behaviour, that the greater the intensity of crowding the greater the degree of activation of the beetles, resulting from numerous physical contacts, and that a certain amount of the latter provides a stimulus for, if not an essential preliminary to, the act of copulation. This, together with the decreased opportunity for copulation, so that when it does occur it is too late in the life of the individual to result in normal productivity, seems to be the explanation of the existence of an "optimum" density for reproductive rate.

Effective Factor under Conditions of "Moderate Crowding."—Considering now the densities within the range indicated by the optimum and the point of flexion of the curve in fig. 3, *i.e.*, under conditions of moderate crowding, we are confronted with the greatest difficulty of all in explaining the reduced rate of reproduction, especially in view of the results derived from the experiments involving the use of "dummy" grains and the addition of *Sitotroga*. In those experiments, it will be recalled, that while the presence of the moths had the effect of causing a significant reduction in the reproductive rate of the weevils, the addition of glass beads to the environment failed to do so ; and further, although the moths did bring about a reduction, the latter was less than that resulting from the presence of a similar number of weevils. Thus whatever the ultimate nature of the factor responsible for these reactions it is not species specific, so that the effect can be brought about through the presence of other species in the environment, despite the absence of direct competition for food or oviposition space. The logical conclusion is, therefore, that the so-called "density factor" has not been reduced to its simplest terms, and is indeed a multiple one.

With this reservation, we can accept Pearl's explanation of the phenomenon as being the result of adverse effects upon the reproductive system induced by

factors which must be termed, in the present state of our knowledge, "psychological." That is, to say that such an effect actually takes place would appear to be correct, but it is not the whole story. Moreover, the so-called "psychological" effect almost certainly results from the repeated stimulation of excessively numerous physical contacts. Confirmatory evidence of such an adverse effect is derived from a consideration of Bilski's (1921) work on the effect of crowding upon the growth rate of tadpoles, which he found to be a function of the group stimulation possibilities.

Although empirical, Bilski's formula conformed to his results with tadpoles as well as those of several others working with different animals, and may be written thus: $Y = K(\sqrt{x}/x - 1)$, where Y represents size of animal and x the population density. Now, this same formula agrees reasonably well with the writer's observations on the effect of population density upon reproductive rate, within the range of "moderate crowding," but does not hold good at other densities. Moreover, the facts concerning the reproductive rate within this range of crowding are not in consonance with the possibility of a purely mechanical effect such as interruption with oviposition or copulation, as derived from a study of the probability of physical contacts in the different densities. Hence it seems fairly certain that while the primary stimulus is mechanical, in that it is a contact stimulus, it operates organically through a psychological reaction which adversely affects the physiological processes of reproduction. In certain species the near approach of another organism may be sufficient to set up a reaction.

Effective Factor under Conditions of "Intense Crowding."—As regards the explanation of the reduced reproductive rate at densities below the point of flexion of the curve in fig. 3, and which, for convenience of reference, may be termed "condition of intense crowding," the fact that Bilski's formula no longer holds good is in itself suggestive of the preponderating influence of some other unknown factor. Further, since the reduction in number of progeny under conditions of "intense crowding" is approximately in inverse proportion to the chances of physical contacts it is not unreasonable to assume that the effective factor is the mechanical interruption of the females in their attempts to oviposit, together with, but to a lesser degree, interference in the act of copulation. Direct observation reinforces these conclusions. Under these conditions the "psychological" factor cannot make its effect visible.

Finally, judging from the number of grains which showed no exit holes, the percentage of grains which contain more than one weevil increases with the intensity of crowding. But this increase is sufficiently small to make it certain

that the female possesses the power of distinguishing grains which already contain an egg from those which do not, but that this capacity for discrimination is impaired by excessive crowding. In this light, the behaviour of the weevils in the experiments with the "dummy" grains becomes intelligible in that they would tend to avoid their immediate vicinity (being of no value for food or oviposition) and so leave the possibilities of contact stimulation much as before, at least under conditions of "moderate crowding," when the presence of the "dummies" had least effect on the behaviour of the insects.

Possible Significance of Crowding in Relation to Certain Phenomena in Nature.—Having thus presented the evidence for, and mode of operation of, a true density effect, produced under the experimental conditions of the laboratory, one may now legitimately ask the question, what is the general biological significance of such an effect, if any? To mention only a few instances in which this "space factor" may play the dominant, although unsuspected, role, there is the problem of insect migration. The causes underlying the urge to migrate are extremely obscure, and it has recently been suggested by Williams (1930) that "a form of ecstasy or hysteria" is the responsible factor, but it is not inconceivable that the fundamental factor is the psychological effect of excessive contact stimulation due to crowded conditions of existence, and it should be borne in mind that this factor comes into operation long before there is any real or apparent shortage of food supply. There is also the problem of certain obscure phenomena associated with the epidemiology of insect outbreaks in which the "space factor" would also seem to be responsible, particularly in the initial stages of introduction of a pest to a new country, and again just prior to the subsidence of an outbreak.

The fact that this density effect gives the appearance, superficially, of a "space consciousness" on the part of the insects, recalls to mind the statement by Elton (1930) that "migratory behaviour seems to require the existence of a definite awareness of inharmonious relations with the environment." This statement, which Elton says he owes to Farthing of McGill University, though plausible, really serves to cloak our ignorance regarding the factors responsible for migratory behaviour. Moreover, the theme is not new, since essentially the same idea underlies Lowne's statement (1890) that the female *Calliphora erythrocephala* consciously adapts the size of her egg cluster to the available oviposition surface of the medium. Such postulated attributes of the lower animals must be regarded with suspicion, especially where more definite factors would seem to be responsible. That contacts do play an important role in

the lives of the lower animals is supported by Wheeler's belief that the ant's world is largely one of contact-odor shapes.

It would appear, therefore, that natural populations automatically check their own increase by virtue of this density effect, and that the organism itself imposes the ultimate limit to its own abundance when all other factors (biotic and physical) normally inhibiting population increase, have failed.

IV. *Mathematical Representation of the Data.*

On account of the great definiteness with which the density effect operates, together with the reasonable accuracy of the data obtained as a result of using an inbred strain of weevils, this investigation would not be complete without some attempt to place the results on a mathematical basis. Farr in 1843, developed an equation to describe the relation between the density of human population and the death-rate, while Pearl and Parker (1922) found that essentially the same equation (to which a slight curve fitting refinement was added) expressed the relation between crowding and reproductive rate in *Drosophila*. This old law of Farr states that $R = cD^m$, where R is the death-rate, D the population density per unit area, and c and m are constants. Now, the writer finds that this equation also describes the effect of crowding on reproductive rate of *Calandra*, but that the values of the constants only hold good over a certain range of population density, when they give place to other values, indicating a change in the law at certain degrees of crowding. This conforms with the evidence already presented in regard to the different effective factors operating at different densities. When presented in logarithmic form the above equation becomes

$$\log Y = \log a + b \log X,$$

where Y represents progeny per weevil and X the number of grains per weevil. From this we can tell by inspection that if the data conforms to this formula we should obtain a straight line when $\log Y$ is plotted against $\log X$. Hence, we have at hand a convenient method for testing the accuracy of our experimental data and their degree of conformation to the theoretical relationship represented by the formula.

The plot of the two variables on the doubly logarithmic scale is presented in fig. 4, in which the black dots represent the actual data, and to which the straight lines have been fitted according to the "method of averages." It is quite apparent from the diagram that a single straight line does not describe the situation, but with two straight lines the fit is extraordinarily good. Since b

gives the slope of the line and a its intercept on the Y-axis, it follows that the values of the constants change at the point of intersection of the straight lines and that this point of intersection corresponds to the point of flexion of the curve in fig. 3. Within the range $X = 0.25-9$ (wheat grains per weevil) the parameters a and b have values of 0.19 and 1.66 respectively, while from 9.1-200 the corresponding figures are 1.38 and 0.55. For values of X above 200, the slope of the line is reversed and b becomes negative, as a result of the decreasing rate of reproduction with increasing space above this degree of crowding. On the other hand, for values of X below 0.25 the slope of the

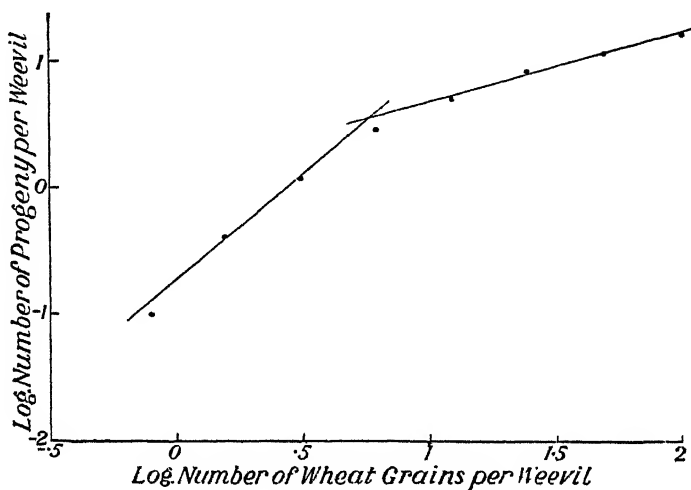


FIG. 4. $\log Y = \log a + b \log X$.

line is almost vertical so that b becomes very large, and since the logarithm of these values of X is negative, the quantity $(\log a + b \log X)$ diminishes very rapidly, and indeed, for all practical purposes, becomes zero when X has a value of 0.125. That is to say, the weevils practically cease to reproduce when there are four females to every grain of wheat, and the population consists of equal numbers of both sexes. The exponent b describes the biological law in relation to reproductive rate and population density, and may be used as an index of the same, in a manner corresponding to that of a "temperature coefficient."

The author is indebted to Professor Crozier of Harvard University for laboratory facilities and helpful advice throughout the progress of the work; also, to Dr. C. B. Williams and to Dr. A. D. Imms, F.R.S., for reading the manuscript.

V. *Summary.*

(1) The present paper confirms in quantitative terms a suggestion previously put forward by the writer, that the effects of population density are of greater significance than generally realised.

(2) A survey of investigations on the subject shows that considerable confusion prevails on account of the experimental difficulties involved in the analysis of a true density effect.

(3) Analysis of existing data, combined with laboratory experimentation upon different insects, demonstrate the great definiteness of operation of the "space factor"; also, the existence of an "optimum" density for rate of reproduction.

(4) The factors responsible, at different densities, for the reduction from the maximum reproductive rate, have been examined together with their possible significance in relation to certain phenomena in Nature associated with migration and the epidemiology of insect pests.

(5) The results have been placed on a mathematical basis, in which an empirical formula, originally used by Farr, has been adapted, following the work of American investigators, to describe the relation between population density and rate of reproduction, thus: $\log Y = \log a + b \log X$, where Y represents progeny and X the space per individual, and a and b are constants.

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The Production of Cancer by Pure Hydrocarbons.—Part I.

By J. W. COOK, I. HIEGER, E. L. KENNAWAY, and W. V. MAYNEORD.

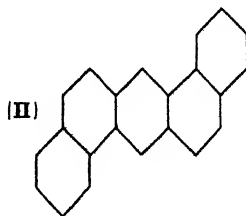
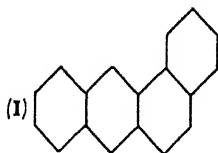
(From the Research Institute, The Cancer Hospital (Free), London).

(Communicated by Sir Henry Dale, Sec.R.S.—Received May 12, 1932.)

[PLATES 15–17.]

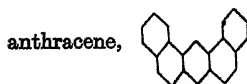
Introduction.

Hydrocarbons are known to have the power to produce cancer in mice, for the carcinogenic mixtures obtained by heating acetylene or isoprene in an atmosphere of hydrogen (Kennaway, 1924, 1925) can consist only of hydrocarbons. Moreover, the powerful fluorescence of these and other carcinogenic mixtures (*e.g.*, gas-works tar; shale oil; heated petroleum; products of the action of heat upon cholesterol, yeast, skin, muscle, and hair, and of the action of aluminium chloride upon tetralin) suggests that such hydrocarbons are of the polycyclic aromatic type. These mixtures gave a characteristic three-banded fluorescence spectrum, which resembled in character that of 1:2-benzanthracene. This significant observation led to the examination for carcinogenic action of a large number of pure polycyclic aromatic hydrocarbons of known molecular structure, particular attention being paid to compounds related to 1:2-benzanthracene (I). One such hydrocarbon, 1:2:5:6-dibenzanthracene (II), was prepared by the method of Clar (1929, *a*), and gave positive results.



When the first four cancers produced in mice by this substance had been obtained, a preliminary account of the experiments was published (Hieger, 1930; Kennaway, 1930).^{*} We now have specimens of 102 cancers and 31

^{*} In this paper the four cancers were attributed to the action of 1:2:7:8-dibenz-



for they were produced by the compound so described by Clar (1929, *a*), who obtained it by pyrolysis of 2-methyl-1:1'-dinaphthyl ketone. It was

papillomas produced by this compound and its simple methyl derivatives,* while various other compounds have given negative results. The production of tumours of subcutaneous tissue by injection of fatty media containing 1:2:5:6-dibenzanthracene has been recorded elsewhere (Burrows, Hieger and Kennaway, 1932), and the numerous tumours of the peritoneal cavity which have been produced by similar methods are described by Burrows (1932).

Although the investigation of fluorescence spectra has led us to the discovery of the carcinogenic properties of 1:2:5:6-dibenzanthracene, we are not prepared to assert that the constituents of cancer-producing mixtures to which their carcinogenic activity is due are identical with those which produce the characteristic spectrum, nor do we express any opinion on the question whether the cancer-producing constituents of such mixtures are of the dibenzanthracene type. These matters are still under investigation; for the present it suffices to say that we have examined with negative results a synthetic hydrocarbon (phenanthrafluorene), and also fractions of crystalline coal-tar products, which give spectra resembling those of typical cancer-producing mixtures more closely than does that of 1:2:5:6-dibenzanthracene. On the other hand, crystalline fractions have been separated from coal-tar pitch, by a method to be described later, which not only show the typical fluorescence spectrum, but are also strongly carcinogenic.

Technique.

The compounds tested were applied in solution to the interscapular region of mice in the ordinary way; in most cases twice weekly. Details of the solvent used, and of the amount of hydrocarbon added to it, are given in Tables I, II, III and VI. When the whole of the substance added did not dissolve, the concentration of the substance in solution was not known. In the great majority of cases the solvent was benzene, but other media (xylene, tetralin, 1:6-dimethylnaphthalene, liquid paraffin, oleic acid, lard) were used in a few experiments. A concentration of 0.30 to 0.33 per cent. of 1:2:5:6-dibenzanthracene gives a solution which is saturated except in hot weather, subsequently shown by one of us (Cook, 1931, *a*) that this hydrocarbon is actually 1:2:5:6-dibenzanthracene, which was also prepared by Clar by pyrolysis of 2-methyl-1:2'-dinaphthyl ketone. The previously unknown 1:2:7:8-dibenzanthracene has since been synthesised (Cook, 1932), and is being tested for carcinogenic action.

* This material was exhibited at the Annual Meeting of the British Medical Association at Eastbourne in July, 1931, and before the Pathological Section of the Royal Society of Medicine in February, 1932.

and in our later experiments nearly all the solutions of compounds tested were made up at the lower strength (*i.e.*, 0.3 g. substance to 100 c.c. benzene), whether this gave a saturated solution or not. It is impossible to attain to any satisfactory uniformity in this matter of solvent, because all the compounds that one desires to test are not sufficiently soluble in any one solvent which is at the same time suitable for application to the skin of a mouse. Thus, many of the higher hydrocarbons represented in Tables I, II and VI are much more soluble in xylene, or in tetralin, than in benzene, but the two former liquids are undesirable because they produce roughness and irritation, and occasionally minute papilloma-like structures in the skin. The few comparative data that are to be found in the tables (see 1 : 2 : 5 : 6-dibenzanthracene in liquid paraffin, 1 : 6-dimethylnaphthalene, and lard, Table III) suggest that the solvents other than benzene which we happened to try are not superior to benzene for purposes of tumour production. The slight solubility of many of the higher hydrocarbons, especially of those containing more than five benzene rings (Table VI), makes all tests upon them unsatisfactory.



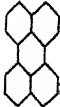
Thus all lines of cancer research which depend upon the response of the skin to chemical agents are beset with possibilities of error which cannot be avoided. By the general use of a single solvent (*e.g.*, benzene), one can at least secure uniformity in any influence of the solvent itself. These considerations do not lessen the value of positive results, but they do show the need for caution in accepting negative results. Possibly, the best solvent for such comparative experiments would be the fat of mouse skin, if it could be obtained in sufficient quantity (*cf.* Watson, 1931). In this paper we can do no more than record the observations which we have made; we cannot say what results might be obtained under other conditions.

Hydrocarbons Tested for Carcinogenic Action.

Negative results from the application to mice of eight polycyclic aromatic hydrocarbons (acenaphthene, fluorene, anthracene, β -methylantracene, phenanthrene, retene, picene, truxene), and of the nitrogenous compounds carbazole and acridine, have been recorded in an earlier paper (Kennaway, 1930).

The hydrocarbons dealt with in the present paper may be classified according to the number of benzene rings contained in them. Two compounds present in coal-tar which do not fall into this category are diphenylene oxide and fluoranthene (Table I).

Table I.—3-Ring, 4-Ring and 5-Ring Compounds.

	Solution.			Mice.			Tumours.	
	Solvent.	Concentration per cent.	Saturated	Initial number.	Alive after—		Epithelioma.	Papilloma.
					6 months.	1 year.		
Diphenylene Oxide 	Benzene	2.0	—	5	5	4	0	0
Fluoranthene 	"	0.3	—	{ 10 10	4 3	1 3 alive, day 308, negative	0	0
<i>4-Ring Compound.</i>								
1:2-Benzanthracene (Formula I, p. 455)— (1) Not specially purified	"	2.0	—	50	13	2	0	0
(2) Purified by picrate	"	0.3	—	30	10	7	6 alive, day 391, negative	
<i>5-Ring Compounds.</i>								
Perylene 	"	1.0	+	20	14	7	0	0

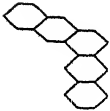
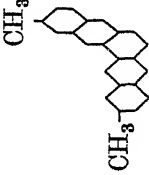
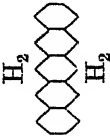
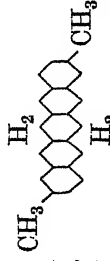
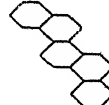
2' : 3'-Naphtha-1 : 2-anthracene  (from Dr. Clar)	0.3	+	20	14	3	561	0	0
7 : 7'-Dimethyl-2' : 3'-naphtha-1 : 2-anthracene  (from Dr. Clar)	0.25	+	20	3	None	350	0	0
Mixture of 2' : 3'-Naphtha-1 : 2-anthracene and 2 : 3 : 6 : 7-Dibenz-9 : 10-dihydro-anthracene, prepared by Clar's method (Clar, John, and Hawran, 1929) 	1.0 to 0.5	+	10	5	2	743	0	0
Mixture of 7 : 7'-Dimethyl-2' : 3'-naphtha-1 : 2-anthracene and a dimethyl derivative of 2 : 3 : 6 : 7-Dibenz-9 : 10-dihydroanthracene— (1) Prepared by Clar's method (<i>loc. cit.</i>) (2) From Dr. Clar 	0.7 to 1.0 0.25	+	10 5	7 5	1 3	732 1 alive day 685, negative	0	0
1 : 2 : 7 : 8-Dibenzphenanthrene (Picene) 	0.5 0.2	+	30 10	9 6	4 2	438 538	0 0	? 1 0
Tetralin Benzene								

Table II.—1 : 2 : 3 : 4-Dibenzanthracene, 2' : 3'-Naphtha-1 : 2-phenanthrene, and 2' : 3'-Naphtha-2 : 3-phenanthrene.

	Solution.		Mice.			Tumours.			
	Solvent.	Concen- tration per cent.	Saturated	Initial number.	Alive after—		Day of death of last mouse.	Epi- thelioma.	Papil- loma.
					6 months.	1 year.			
2' : 3'-Naphtha-1 : 2-phenanthrene (from Dr. Clar) (Formula IV) Series 1	Benzene	0.15	+	5	4	2	478	0	0
2' : 3'-Naphtha-2 : 3-phenanthrene (from Dr. Clar) (Formula V) Series 1	"	0.25	+	5	3, then made up to 5	5	523	0	0
2' : 3'-Naphtha-2 : 3-phenanthrene, m.p. 256°-259°, prepared by the method of Cook (1931, b)— Series 2	"	0.3	+	5	4	None	340	0	0
" 3	"	0.3	+ 40°	10	8	1	1 alive, day 515, negative		
Crude higher-melting isomers of 1 : 2 : 3 : 4-Dibenzanthracene— Series 1	"	0.66	+	10	5	2	779	0	0
" 2	"	0.66	+	5	5	2	556	0	0
" 3	"	0.66	+	40	11	7	1 alive, with pa- pilloma, day 771		
" 4	"	0.66	+	50	26	19	739	0	0
Impure 1 : 2 : 3 : 4-Dibenzanthracene (Formula III). Series 1	"	1.0 per cent. + 0.3 per cent. — 0.25 and 0.3	}	130	1	None	252	0	0
" 2	"			7	3	709	1	0	
" 3	"			20	4	458	0	0	
" 4	"			20	9	756	1	0	
" 5. Crystals melting at 183°-189°, 194°-198°, and 203°-204°*	"			10	5	733	1	0	
				70				3	

* Clar (1929, a) gives the m.p. of 1 : 2 : 3 : 4-dibenzanthracene as 196°-197°, but in a written communication to us gives the figure as 205°. Our sample m.p. 203°-204° was obtained by purification through the picrate.

There are six possible 4-ring aromatic hydrocarbons composed entirely of benzene rings, namely, 1 : 2-benzanthracene, 2 : 3-benzanthracene (naphthacene), chrysene, triphenylene, pyrene and 3 : 4-benzphenanthrene (Cook 1931 c). All these are, or have been, under test here for carcinogenic action. In the present paper only the experiments with 1 : 2-benzanthracene will be dealt with. A preliminary series of 50 mice painted with this hydrocarbon (2 per cent. in benzene) gave one transient papilloma only; the last mouse died on the 574th day. A later series of 30 mice (Table I), of which seven have lived for more than one year, have up to the time of writing given no tumours.

5-Ring Compounds.

Of the fifteen possible 5-ring compounds composed entirely of six-membered aromatic rings, ten are known, and all of these are, or have been, under test here for carcinogenic action (Tables I, II, III). These compounds are as follows :—

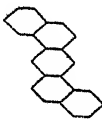
1 : 2 : 3 : 4-Dibenzanthracene.	3 : 4 : 5 : 6-Dibenzphenanthrene.†
1 : 2 : 5 : 6-Dibenzanthracene.	2' : 3'-Naphtha-1 : 2-anthracene.
1 : 2 : 7 : 8-Dibenzanthracene.	2' : 3'-Naphtha-1 : 2-phenanthrene.
2 : 3 : 6 : 7-Dibenzanthracene.	2' : 3'-Naphtha-2 : 3-phenanthrene.
1 : 2 : 7 : 8-Dibenzphenanthrene (picene).	Perylene.

In addition, the 3'-methyl derivative of 1 : 2 : 5 : 6-dibenzanthracene (Table III), and preparations containing the 7 : 7'-dimethyl derivative of 2' : 3'-naphtha-1 : 2-anthracene, and dimethyl-dihydro- and dihydro- derivatives of 2 : 3 : 6 : 7-dibenzanthracene (Table I) have been tested.

Of these, picene, which is very insoluble, has been applied in three media to 90 mice (a preliminary series in which the hydrocarbon was applied in dermoid oil to 50 mice is not included in the table); no tumours, except one doubtful papilloma, have been obtained. The tests of 1 : 2 : 7 : 8-dibenzanthracene, 3 : 4 : 5 : 6-dibenzphenanthrene, and 2 : 3 : 6 : 7-dibenzanthracene have not been in progress very long, and hence these experiments are not included in the tables. Chemically, 2 : 3 : 6 : 7-dibenzanthracene is the most active of these hydrocarbons. It is a deep blue substance, which has the properties of a divalent free radical (Clar and John, 1930). Results obtained with this compound must be accepted with reserve, as the substance is so very sparingly soluble that it is probably impossible to apply it in a concentration

† m.p. 177°–178°, prepared by J. W. Cook (unpublished experiments).

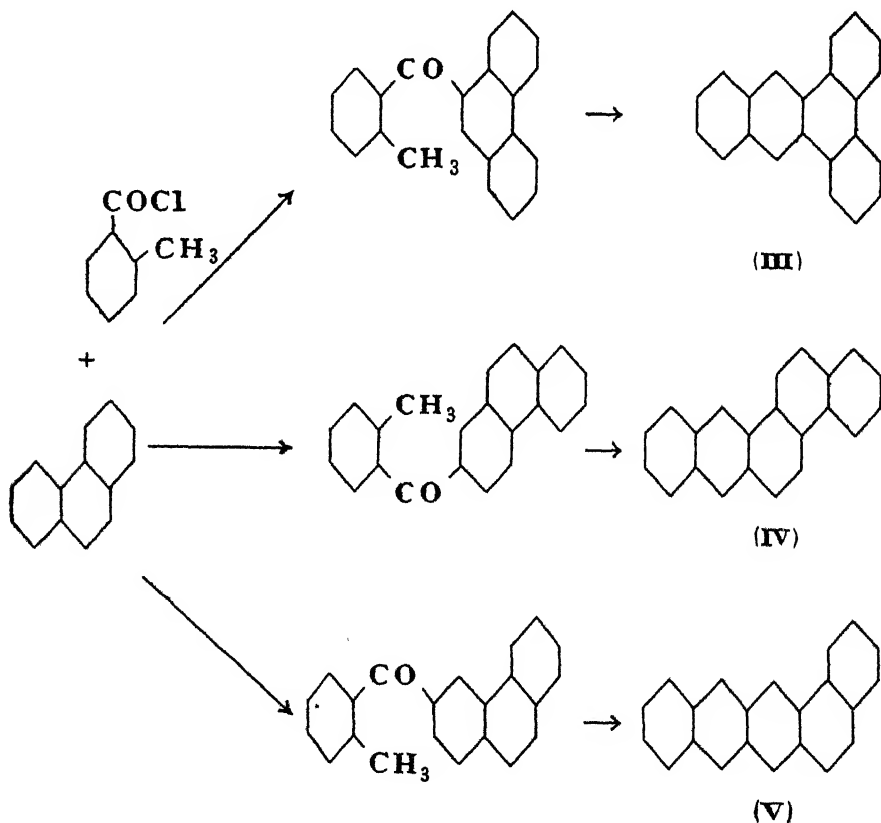
Table III.—1 : 2 : 5 : 6-Dibenzanthracene and 3'-Methyl-1 : 2 : 5 : 6-dibenzanthracene.

Series.	Solution.	Mice.			Tumours.					
		Solvent.	Concen- tration. per cent.	Saturated Number.	Alive after		Epi- thelioma.	Papil- oma.		
					6 months.	1 year.				
1 : 2 : 5 : 6-Dibenzanthracene.										
										
<i>Preparation.</i>										
1	Mother-liquor of crystals melting at 260°-260.5°	Benzene	2.0	+	10	6	None	278	4	0
2	Same material as Series 1, and crystals melting at 257°-259°	"	0.3	-	20	12	1	377	10	1
3	Various preparations not specially purified	"	1.0	+	10	3	2	536	3	0
4	Same as Series 3	"	1.0	+	20	10	None	333	5	4
5	"	"	1.0	+	20	9	"	339	2	1
6	"	"	1.0	+	10	2	"	359	1	0
7	"	"	0.3	-	20	15	"	333	11	1
8	M.p. 257°-259°	"	0.3	-	20	9	"	329	7	2
9	Same as Series 3	"	0.3	-	20	19	"	343	14	1
10	Purified by passage through picrate, and then by H ₂ SO ₄ . M.p. 259°-260°	"	0.3	-	10	6	1	433	1	4
11	Same as Series 9	"	0.3	-	20	11	None	336	8	1
12	Purified by H ₂ SO ₄ , and then by passage through picrate. M.p. 259°-260°	"	0.3	-	10	5	"	343	1	2
13	Same as Series 11	"	0.3	-	13	7	"	312	2	3
14	"	"	0.03	-	20	10	6	436	4	1
15	"	"	0.3	-	10	5	None	336	4	0
Purified by passage through picrate. M.p. 258.5°-260°		"	0.3	-	233	129			77	21

sufficiently high to show any activity. The solvent used for the application of this compound to mice is lard at 40°, to which it imports a blue colour.

1 : 2 : 3 : 4-Dibenzanthracene, 2' : 3'-Naphtha-1 : 2-phenanthrene, and 2' : 3'-Naphtha-2 : 3-phenanthrene.

A mixture containing these three hydrocarbons is readily prepared by the pyrolysis of a crude mixture of ketones formed by interaction of *o*-toluoyl chloride and phenanthrene (Clar, 1929, *a, b*), and because of their close relation to 1 : 2 : 5 : 6-dibenzanthracene we have applied these hydrocarbons to a larger number of mice (200) than was practicable in the case of some of the other 5-ring compounds. According to Clar, the course of the reaction is depicted by the scheme :—



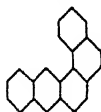
III.—1 : 2 : 3 : 4-Dibenzanthracene m.p. 205°.

IV.—2' : 3'-Naphtha-1 : 2-phenanthrene m.p. 293°–294°.

V.—2' : 3'-Naphtha-2 : 3-phenanthrene m.p. 263°–264°.

Of these three hydrocarbons 1:2:3:4-dibenzanthracene (III) and 2':3'-naphtha-1:2-phenanthrene (IV) were isolated by Clar by fractional crystallisation, and the presence in the mixture of 2':3'-naphtha-2:3-phenanthrene (V) was shown by oxidation of a fraction to a diquinone which gave a phenazine identical with that obtained from a sample of (V) prepared by another method. The preparation of (V) by an independent method by one of us (Cook, 1931, b) confirms the structure assigned by Clar. The constitution of 1:2:3:4-dibenzanthracene (III) was established by Clar by its oxidation to a monoquinone which had the properties of an anthraquinone derivative. With regard to (IV) this was isolated by Clar by repeated ("nearly 20") crystallisations from xylene. We have found that this procedure can be considerably simplified by making use of the observation mentioned below, that (V) reacts readily with maleic anhydride. A fraction, m.p. 265–280° obtained by three recrystallisations of the crude mixture of hydrocarbons from xylene, was heated for an hour in xylene solution with maleic anhydride. The 2':3'-naphtha-1:2-phenanthrene (IV) was then readily obtained pure by steam distillation of the xylene, in presence of alkali, followed by recrystallisation of the insoluble residue from xylene. The structure assigned by Clar to this hydrocarbon is probably correct, but it should be pointed out that the possibility that the

substance is 2':3'-naphtha-3:4-phenanthrene



is not definitely excluded.

Recently Clar and Wallenstein (1931) claimed to have synthesised this last-named hydrocarbon, but the properties were curiously similar to those of 1:2:5:6-dibenzanthracene, the formation of which might be anticipated by the reaction which they used, and in a private communication Dr. Clar agreed that the substance really was 1:2:5:6-dibenzanthracene. The experiments carried out upon mice with these compounds are recorded in Table II.

In all, 13 series of mice comprising 200 animals have been painted with these mixtures and with more or less pure preparations of these three hydrocarbons, but only three tumours were obtained (*cf.* 98 tumours from 233 mice painted with similar solutions of 1:2:5:6-dibenzanthracene), and the one surviving mouse, of the 105 painted with the crude higher-melting isomers, now bears, 771st day, a papilloma. The results obtained with 1:2:3:4-dibenzanthracene are shown in the graph, fig. 1, which is given for comparison with those, figs. 3 to 16, representing the effect of 1:2:5:6-dibenzanthracene. These three tumours were (1) two epitheliomas produced by impure 1:2:3:4-

dibenzanthracene (m.p. 183° – 189°), and (2) one epithelioma in a mouse painted chiefly with the purer preparations of the same hydrocarbon (m.p. 194° – 198° , and 203° – 204°), but this tumour appeared after a period when the less pure preparation (m.p. 183° – 189°) had been used for lack of better material. Hence the question, whether 1 : 2 : 3 : 4-dibenzanthracene itself has any carcinogenic

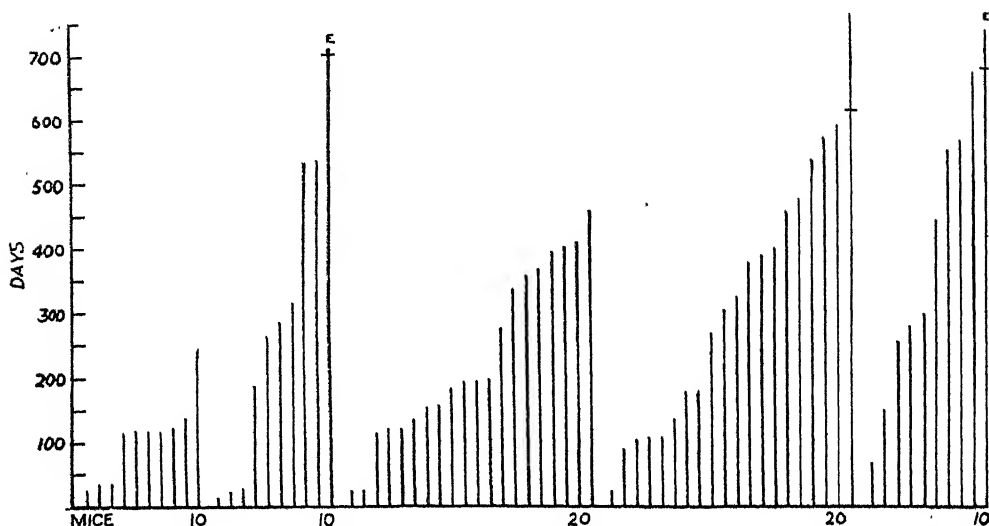


FIG. 1.—Impure 1 : 2 : 3 : 4-dibenzanthracene. [In this and figs. 3 to 19, each vertical line represents the duration of the life of one mouse after the commencement of painting. The transverse mark on some of the lines denotes the day when a tumour was first noticed. The letters at the tops of the lines show the results of microscopical examination of the tumours (E = epithelioma, P = papilloma. K indicates that the mouse was killed to terminate the experiment).]

power at all would have to remain unanswered until further prolonged experiments with very pure preparations had been made. But it is already quite clear that any cancer-producing power of this hydrocarbon must be of a very low order, because the following figures (from Table II) show that it was only the unusual length of life of three mice which allowed any tumours at all to appear.

						Day of appearance of papilloma.	Day of death.
Mouse from Series 2 (Table II)	700	709
„ „ „ 4	619	756
„ „ „ 5	674	733

With these figures the date of appearance of tumours produced by 1:2:5:6-dibenzanthracene should be compared, fig. 2 and Table IV.

Unknown carcinogenic substances are present in many of the impure preparations of these hydrocarbons. Thus epitheliomata appeared in two mice (killed 345th and 392nd day) out of 10 painted with crude ether washings obtained in the purification (Clar, Wallenstein and Avenarius, 1929) of 1':2'-anthra-1:2-anthracene; the pure crystalline hydrocarbon, which is practically insoluble in ether, produced no tumours (Table VI).

Thus three cancers have been produced by impure 1:2:3:4-dibenzanthracene, and two by the unknown impurities removed from 1':2'-anthra-1:2-anthracene, but no cancers have appeared in mice painted with any pure hydrocarbon except 1:2:5:6-dibenzanthracene, its 3'-methyl derivative, and some nearly related compounds described in the next paper (p. 485).

1:2:5:6-dibenzanthracene.

Table III shows that 1:2:5:6-dibenzanthracene was applied to 19 series of mice, comprising 273 animals. The 233 mice treated with solutions of this compound in benzene yielded 77 epitheliomas and 21 papillomas. In all, 107 tumours were obtained

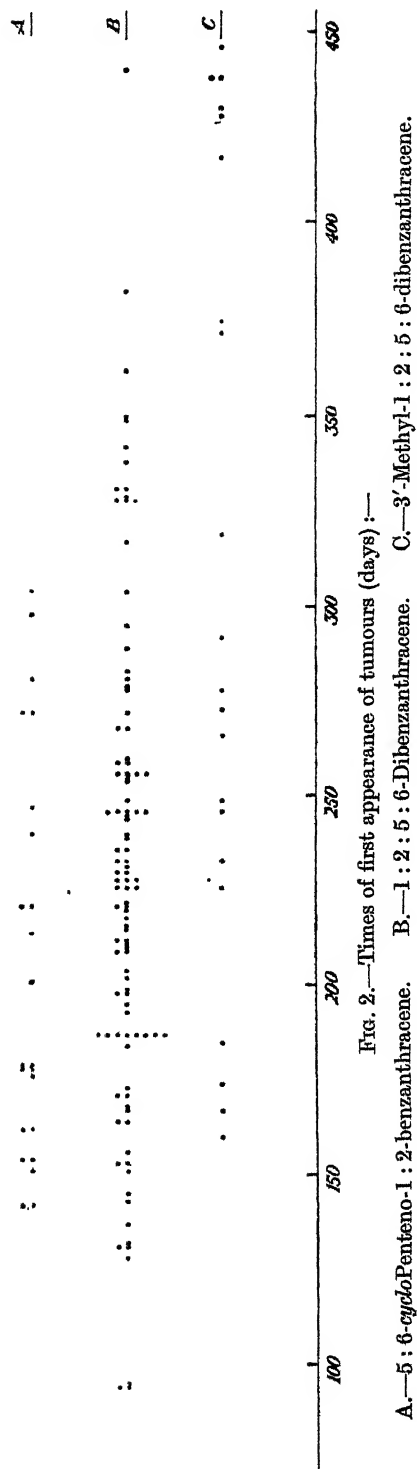


Fig. 2.—Times of first appearance of tumours (days):—

A.—5:6-cycloPenteno-1:2-benzanthracene. B.—1:2:5:6-Dibenzanthracene. C.—3'-Methyl-1:2:5:6-dibenzanthracene.

from 273 mice. The three solvents other than benzene which were used (namely, 1 : 6-dimethylnaphthalene, liquid paraffin, lard, Series 16, 17, 18) did not give any higher incidence of tumours. In Series 19, ischolesterol prepared from lanolin was added to the benzene solution, as it was thought that this compound might have some protective action upon the skin; the incidence of tumours was not lower than in some other series.

[Series of mice now under observation to which 1 : 2 : 5 : 6-dibenzanthracene has been applied in five other solvents (oleic acid, oleic acid and benzene, safrole, isosafrole, mouse fat) have not as yet shown any noteworthy difference in the incidence of tumours from series painted with the hydrocarbon in benzene. Tumours of the skin of the mouse have now been produced by 1 : 2 : 5 : 6-dibenzanthracene in nine different media, namely, the five referred to in the preceding sentence and the four (benzene, 1 : 6-dimethylnaphthalene, liquid paraffin, and lard) named in Table III, and spindle-celled tumours of the rat and mouse have been produced by subcutaneous and by intraperitoneal injection of the hydrocarbon both in lard and in olive oil, making 10 media in all.]

Purification.

The 1 : 2 : 5 : 6-dibenzanthracene used in the earlier experiments, prepared by the method of Clar (1929, *a*) and Fieser and Dietz (1929), was yellowish in colour, in conformity with the statements of these authors, and the colour was not altered by recrystallisation. Nevertheless, it seemed probable that a hydrocarbon of this structure would be colourless when pure, and, if so, it might well be that the carcinogenic action of the substance was due to the coloured impurity. By adopting the following procedure, a specimen of 1 : 2 : 5 : 6-dibenzanthracene was obtained in a high degree of chemical purity, quite free from yellow colouring matter, and it will be seen from Table III that this highly purified hydrocarbon was just as active as the less pure material.

The crude hydrocarbon was twice recrystallised from xylene and 10 grams of the resulting yellow leaflets were converted into the picrate, which was recrystallised from benzene, and decomposed with ammonia; the regenerated hydrocarbon (8.2 g.), which was still yellow, was dissolved in toluene (1800 c.c.), and the cold solution shaken seven times with concentrated sulphuric acid (portions of 50 c.c.). The toluene solution was then washed free from acid, dried with calcium chloride, and concentrated; the pure 1 : 2 : 5 : 6-dibenzanthracene formed colourless plates, m.p. 261°–261.5°, unchanged by further crystallisation from benzene.

This contamination of a solid crystalline hydrocarbon by a persistent impurity finds a parallel in the case of certain higher coal-tar hydrocarbons (anthracene, chrysene). When isolated from the tar these are coloured yellow, and the colour may be removed by suitable chemical treatment. A review of recent literature showed that "chrysogen," the coloured impurity in these hydrocarbons (Fritsche, 1862, 1866), could be identified with 2:3-benzanthracene, which is an orange hydrocarbon (see, for example, Capper and Marsh, 1925; Rădulescu and Bărbulescu, 1929; Fieser, 1931). The isolation of 2:3-benzanthracene from coal-tar does not appear to have been recorded, although Morgan (1928) has reported the isolation of what appears to be a dimethyl derivative from low temperature tar.

In support of the suggestion that "chrysogen" is 2:3 benzanthracene, it has been found that 2:3-benzanthracene has the property of adhering to colourless hydrocarbons, for if anthracene, chrysene, or 1:2:5:6 dibenzanthracene were crystallised from benzene containing a little 2:3-benzanthracene, the crystals were tinted yellow and the colour could not be removed by recrystallisation.

The substance which is responsible for the yellow colour of the crude 1:2:5:6-dibenzanthracene is probably 2':3'-naphtha-2:3-phenanthrene (V), which is a derivative of 2:3-benzanthracene and might be formed by molecular re-arrangement during the production of 1:2:5:6-dibenzanthracene. The colour can be restored to "colourless" 1:2:5:6 dibenzanthracene by addition of this naphtha-phenanthrene during recrystallisation. The method of decolorisation described above depends on the fact that the naphtha-phenanthrene is much more chemically active than 1:2:5:6-dibenzanthracene, and is preferentially attacked by sulphuric acid. A second and more convenient method of decolorisation was suggested by these differences of chemical activity. For when a solution of "yellow" 1:2:5:6 dibenzanthracene (4 parts) in xylene (50 parts) was boiled for an hour with maleic anhydride (1 part), the yellow impurity was completely converted into an alkali-soluble additive compound with maleic anhydride, whereas the 1:2:5:6-dibenzanthracene was scarcely attacked, and could be recovered from the xylene solution in a pure white condition.

Tumour Production in Mice.

Preparations of 1:2:5:6-dibenzanthracene purified by sulphuric acid, either before or after passage through the picrate (Table III), were applied to

six series of mice (Series 9 to 14), and produced 30 epitheliomas and 12 papillomas in 93 animals. This incidence is nearly the same as that produced by the much less pure materials used in Series 1 to 8 (namely, 43 epitheliomas

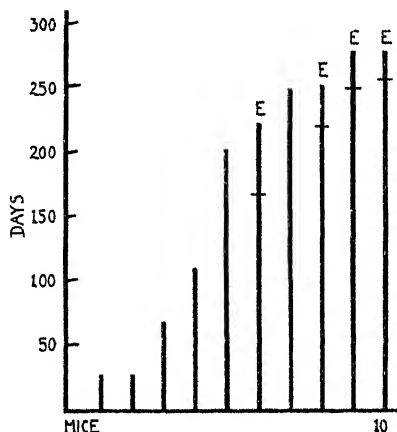


FIG. 3.—1 : 2 : 5 : 6-Dibenzanthracene, Series 1 (Table III).

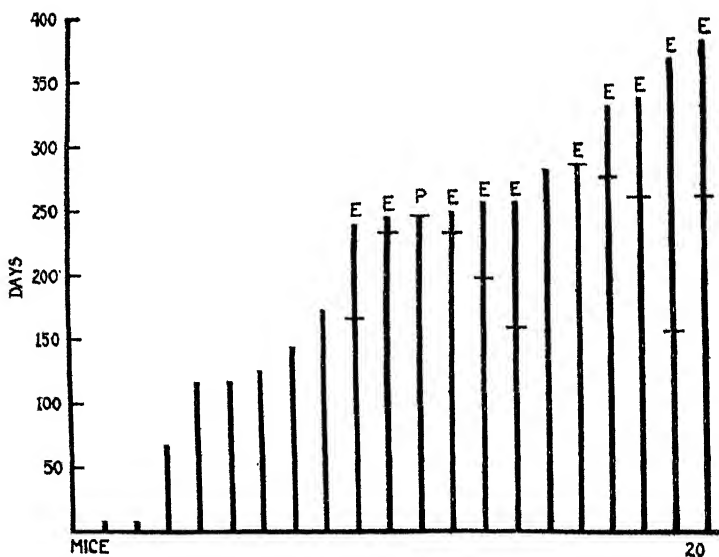


FIG. 4.—1 : 2 : 5 : 6-Dibenzanthracene, Series 2 (Table III).

and 9 papillomas in 130 animals). Therefore it seems certain that the carcinogenic action of 1:2:5:6-dibenzanthracene is not due to any adherent impurity.

The greater number of the series were tested with the solution in benzene containing 0.3 g. crystals in 100 c.c. But a much more dilute solution (0.03 per cent., Series 14, fig. 15) gave 4 epitheliomas and 1 papilloma in 20 mice, of which 10 lived for more than six months. The five tumours appeared after 279, 318, 350, 363 and 384 days. Thus the effect of this dilution was to delay the appearance, rather than to lessen the yield, of tumours. A solution of one-tenth this strength (0.003 per cent.) was applied to a series

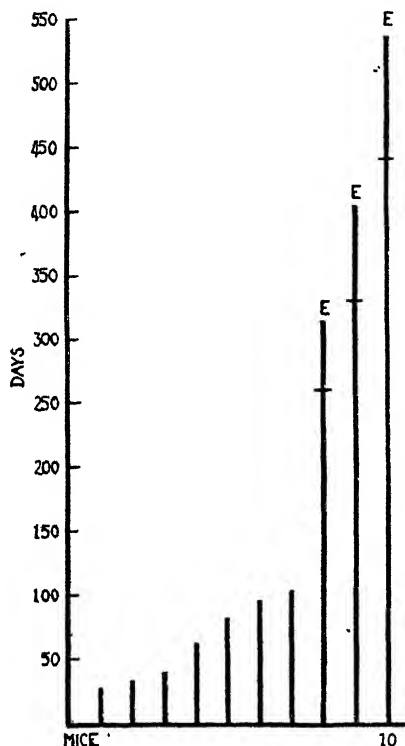


FIG. 5.—1 : 2 : 5 : 6-Dibenzanthracene, Series 3 (Table III).

(not included in Table III) of 20 mice, of which seven lived for more than one year and two for more than one and a half years. On the 659th day the one mouse surviving was found to bear a papilloma which grew rapidly, and had developed into an epithelioma showing considerable invasion of muscle when the animal was killed on the 702nd day, fig. 20, Plate 15.

The recorded times of first appearance of 107 tumours produced by 1 : 2 : 5 : 6-dibenzanthracene are shown graphically in fig. 2. The data are, of course, approximate only, partly because the mice were only examined closely once

or twice weekly, which accounts for the tendency of the dots to appear in batches. Table IV shows that about two-thirds of the tumours appear in the seventh, eighth and ninth months.

Table IV.—Time of Appearance of 107 Tumours of the Skin Produced by 1 : 2 : 5 : 6-dibenzanthracene.

Day.		Number of Tumours.	Per cent.
64 to 184 (120 days)	22	20·5
185 to 285 (100 days)	68	63·5
286 to 442 (156 days)	17	16·0
		107	100·0

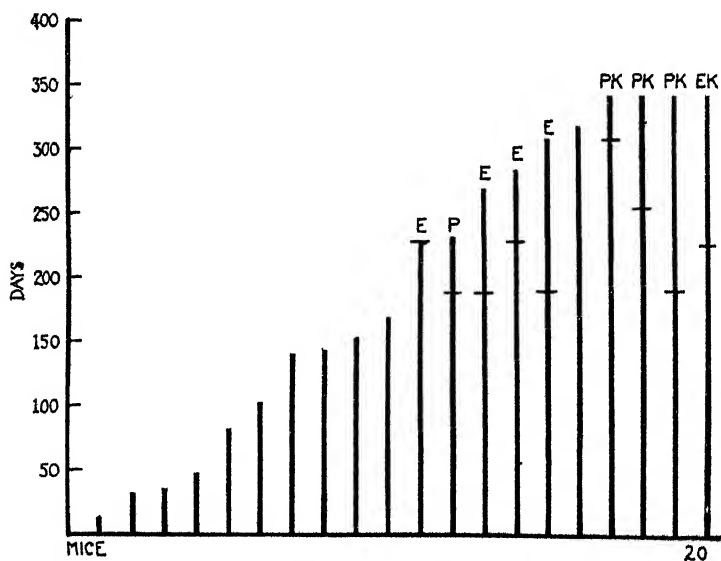


Fig. 6.—1 : 2 : 5 : 6-Dibenzanthracene, Series 4 (Table III).

The earliest tumour appeared on the 64th and the latest on the 442nd day. The variability of the incidence of tumours in the series 1 to 15 is shown in the graphs, figs. 3 to 16. The differences in the yield of tumours in these series are due in part to the variable death-rate in the early months, when no tumours are to be expected, *e.g.*, fig. 8, in part to actual differences in the production of tumours in the later months, which must be attributed to differences in "susceptibility." Thus Series 9, fig. 11, appears to have consisted of mice which were much more susceptible than those of Series 12,

fig. 13. The full series of graphs has been published here in order that these differences may be shown.

The tumours produced by 1 : 2 : 5 : 6-dibenzanthracene present all the usual

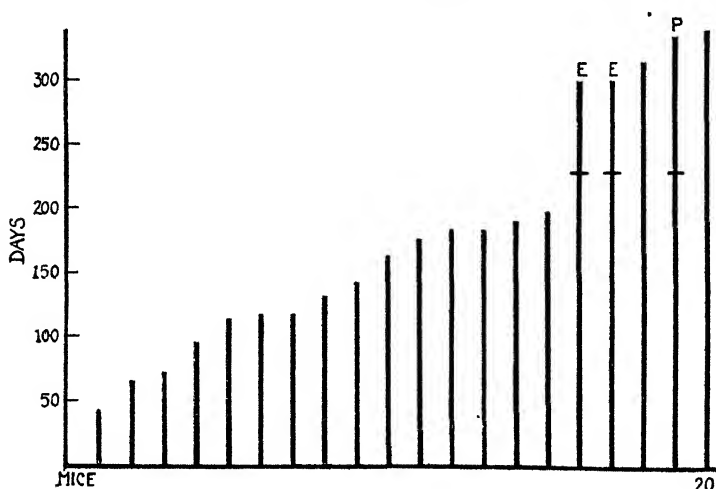


FIG. 7.—1 : 2 : 5 : 6-Dibenzanthracene, Series 5 (Table III).

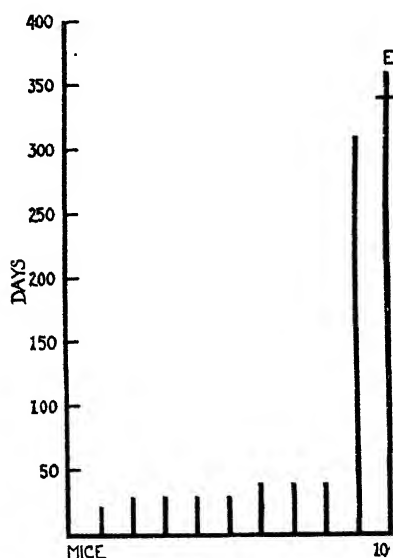


FIG. 8.—1 : 2 : 5 : 6-Dibenzanthracene, Series 6 (Table III).

microscopic appearances of cancer of the skin in mice, figs. 20 to 24, Plates 15 to 17. Tumours showing invasion of voluntary muscle have been classified as epitheliomas, and all others as papillomas, with the exception that,

in a few advanced tumours, when no muscle could be found in the portion taken for section, malignancy has been assumed from the abundance and general character of the growth. Seven of the 30 primary tumours produced by

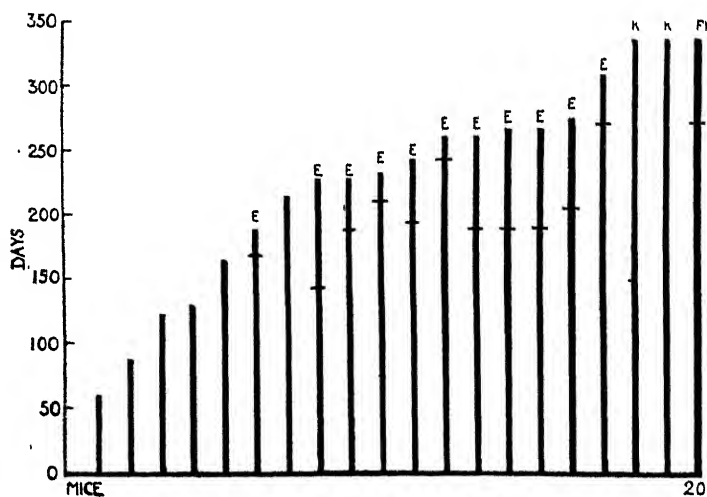


FIG. 9.—1 : 2 : 5 : 6-Dibenzanthracene, Series 7 (Table III).

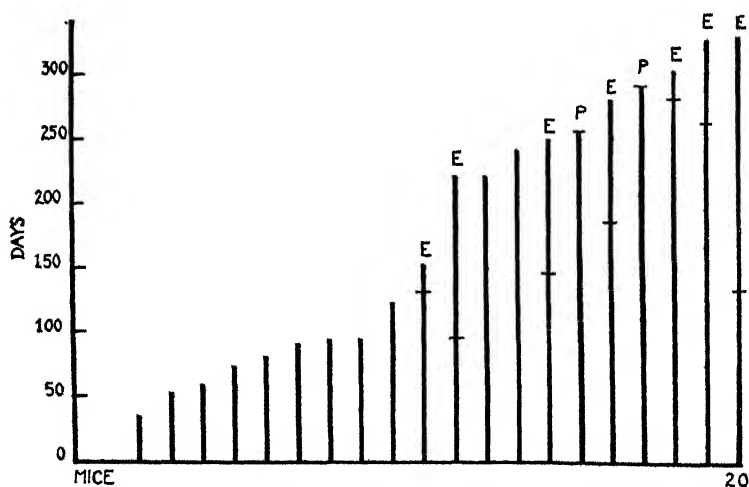


FIG. 10.—1 : 2 : 5 : 6-Dibenzanthracene, Series 8 (Table III).

especially pure material (Series 9 to 14, Table III) show more or less of the spindle-celled character, fig. 21, Plate 16. The great majority of the tumour-bearing mice have been killed as soon as thickening at the base of the growth made it fairly certain that invasion of muscle had occurred ; in this way room

is made more quickly for fresh experiments. This fact, that the tumours were not as a rule allowed to grow until they caused death, is probably in part

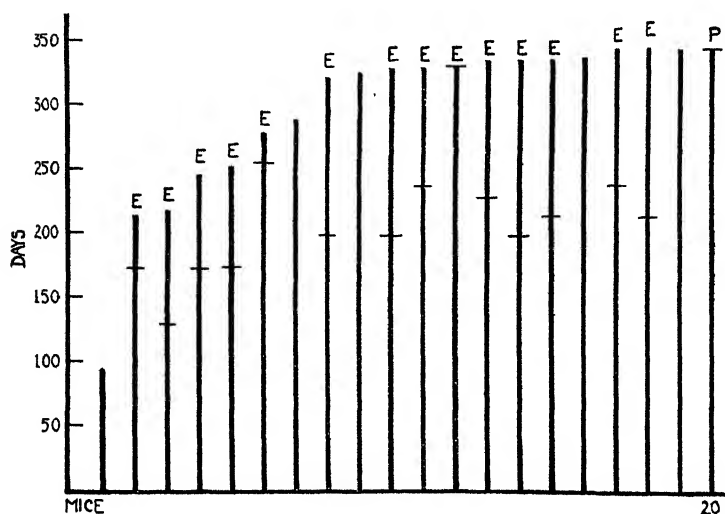


FIG. 11.—Highly purified 1 : 2 : 5 : 6-Dibenzanthracene, Series 9 (Table III).

the reason why glandular metastases were found in one mouse only of those painted with 1 : 2 : 5 : 6-dibenzanthracene. This animal, figs. 22 to 24,

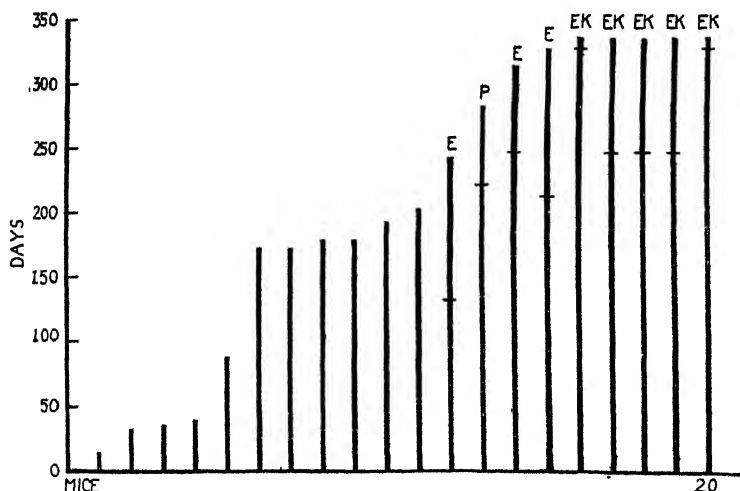


FIG. 12.—Highly purified 1 : 2 : 5 : 6-Dibenzanthracene, Series 11 (Table III).

Plates 16 and 17, was painted with a very pure preparation (Series 9, Table III), and was killed on the 335th day. One of the axillary lymph glands was largely

replaced by epithelioma showing considerable development of horny material. Metastases in lymph glands in a mouse painted with the 2'-methyl derivative,

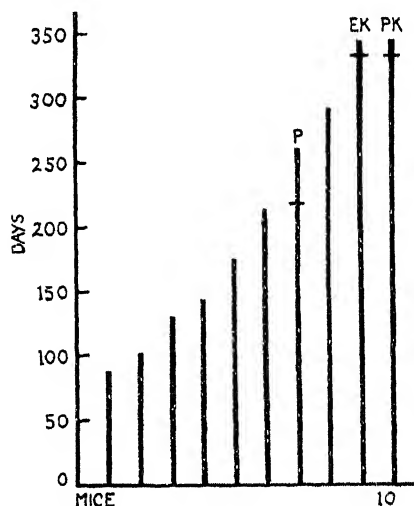


FIG. 13.—Highly purified 1 : 2 : 5 : 6-Dibenzanthracene, Series 12 (Table III).

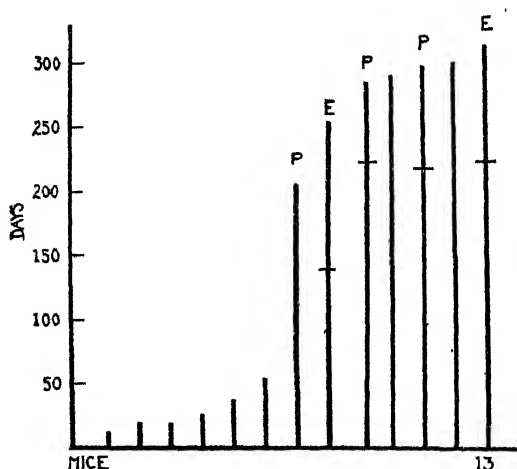


FIG. 14.—Highly purified 1 : 2 : 5 : 6-Dibenzanthracene, Series 13 (Table III).

and several cases of metastasis in lymph glands and lungs in mice painted with 5 : 6-cyclopenteno-1 : 2-benzanthracene will be described in the following paper.

3'-Methyl-1 : 2 : 5 : 6-Dibenzanthracene.

The figures given in Table III provide a comparison of the carcinogenic powers of 1 : 2 : 5 : 6-dibenzanthracene and of its 3'-methyl derivative. The results may be summarised as follows :—

	Number of mice.		Tumours.		
	Initial.	After six months.	Epitheliomas.	Papillomas.	Total.
1 : 2 : 5 : 6-dibenzanthracene in benzene	233	129	77	21	98
3'-methyl-1 : 2 : 5 : 6-dibenzanthracene in benzene	120	70	16	5	21

Roughly then, with the 3'-methyl compound, one-half as many mice gave one-fifth as many tumours.

The recorded times of first appearance of these 21 tumours produced by the 3'-methyl compound are shown in the table below, and graphically in fig. 2. In the table the data are arranged so as to be comparable with those given for 1 : 2 : 5 : 6-dibenzanthracene in Table IV.

Table V.—Time of Appearance of 21 Tumours of the Skin Produced by 3'-Methyl-1 : 2 : 5 : 6-dibenzanthracene.

Day.	Number of Tumours.	Per cent.
161 to 184 (23 days)	3	14·5
185 to 285 (100 days)	8	38·0
286 to 448 (162 days)	10	47·5
	21	100·0

Thus, nearly one-half of the tumours appear in the third period, after the 285th day (*cf.* 1 : 2 : 5 : 6-dibenzanthracene, p. 472 ; only 16 per cent. of the tumours produced by this compound arise in this last period). Thus, the 3'-methyl derivative gives both a lesser, and a slower, yield of tumours than does the parent hydrocarbon ; this is evident if figs. 17 to 19 be compared with figs. 3 to 16.

The two hydrocarbons have approximately the same solubility in benzene at room temperature, so that the lesser carcinogenic power of the 3'-methyl

derivative cannot be attributed to decreased solubility in this solvent. It is, of course, impossible to determine whether different solubilities in the tissues

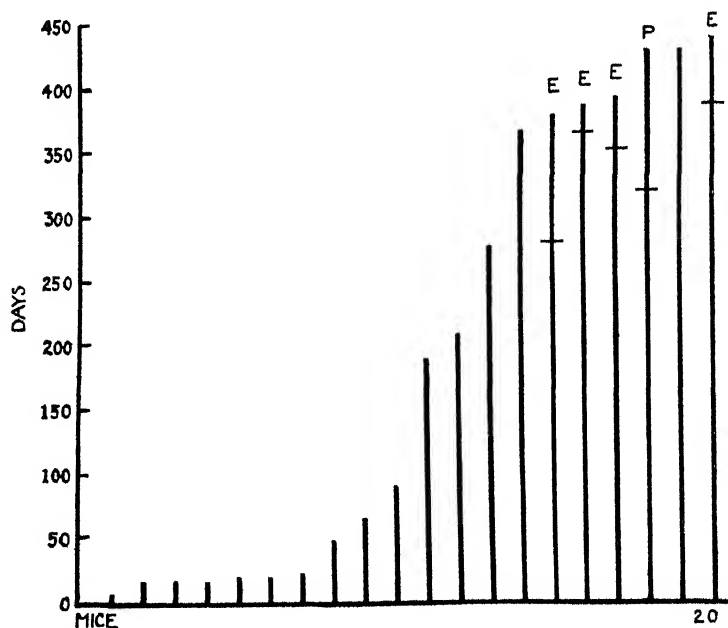


FIG. 15.—Highly purified 1:2:5:6-Dibenzanthracene, 0.03 per cent., Series 14 (Table III).

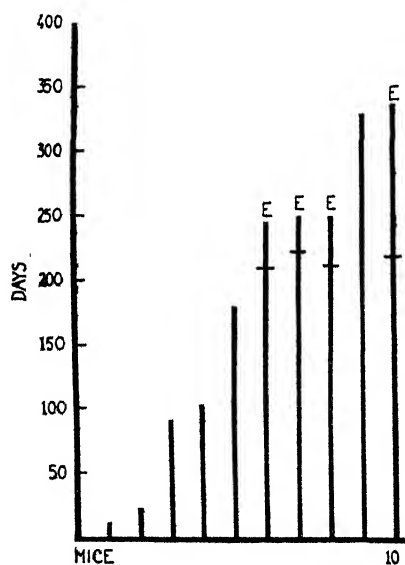


FIG. 16.—Purified 1:2:5:6-Dibenzanthracene, Series 15 (Table III).

of the mouse are ultimately responsible for the observed differences in carcinogenic activity. At any rate, it is clear that the alteration in molecular structure

involved in the attachment of the methyl group to the 1:2:5:6-dibenzanthracene molecule has resulted in a diminution of carcinogenic power.

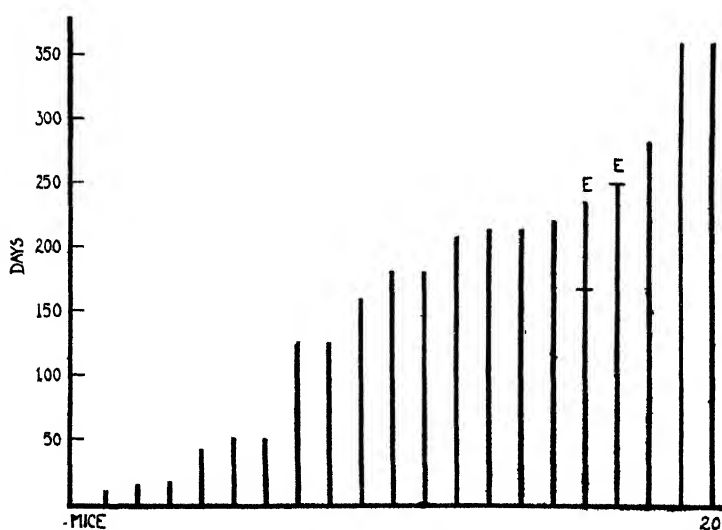


FIG. 17.—3'-Methyl-1:2:5:6-Dibenzanthracene, Series 2 (Table III).

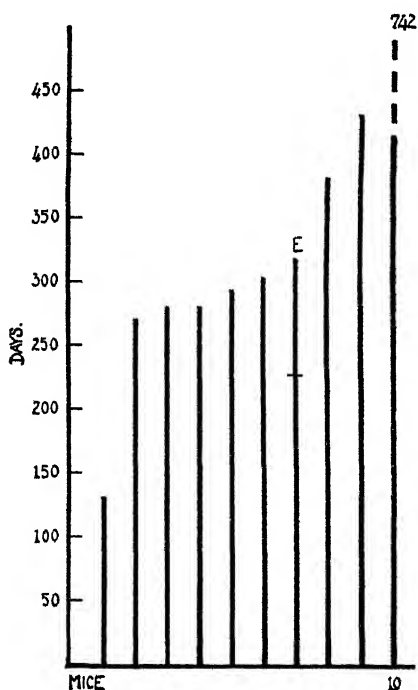


FIG. 18.—3'-Methyl-1:2:5:6-Dibenzanthracene, Series 3 (Table III).

6-Ring Compounds.

Three of these were examined (Table VI). 1:2:3:4:5:6-Tribenzanthracene (Fieser and Dietz, 1929), which is slightly more soluble in benzene at room temperature than 1:2:5:6-dibenzanthracene, is of especial interest

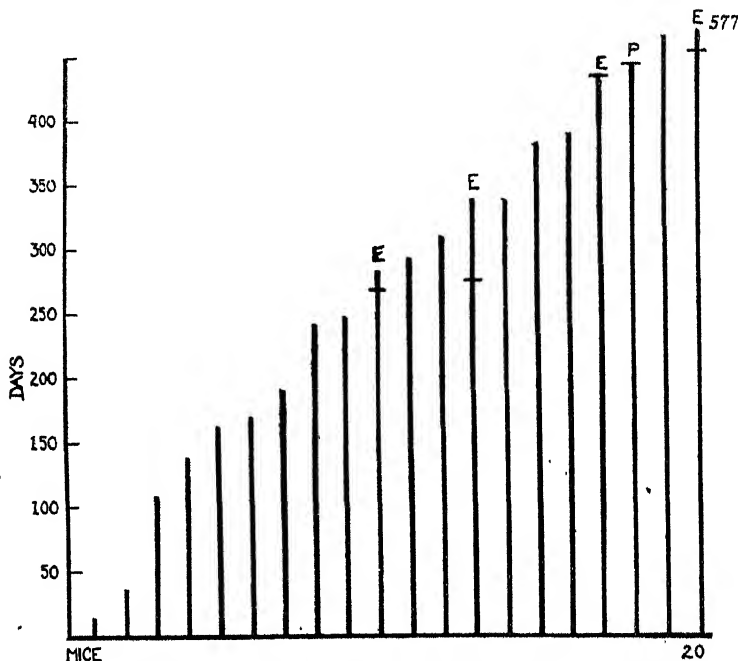


FIG. 19.—3'-Methyl-1:2:5:6-Dibenzanthracene, Series 4 (Table III).

on account of its close relationship to the carcinogenic 1:2:5:6-dibenzanthracene, from which it differs by the addition of a ring in the 3:4 position. This compound gave a transitory papilloma in one mouse (as did 1:2-benzanthracene, see above), but beyond this no tumours were obtained. Thus the further increase of molecular complexity appears largely to have destroyed the carcinogenic activity.

8-Ring Compounds.

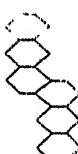
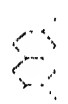
4:5:10:11-Di-(1':2'-naphtha)chrysene (Fieser and Dietz, 1929), which is the compound of greatest molecular weight tested, is of very low solubility, but it is easy to obtain in quantity and was applied in three media (oleic acid, tetralin, benzene).

One of us (I. Hieger) has been working under a grant from the British Empire Cancer Campaign. We are indebted to the Librarian to this Institute, Miss Knox, B.A., for preparing all the graphs illustrating these investigations, and also for the greater part of the work of tabulating the very numerous records of experiments for publication. We wish to thank Dr. E. de Barry Barnett for the gift of valuable samples of pure hydrocarbons. Dr. E. Clar, of Milan, has very generously sent to us many specimens of compounds, which are duly recorded in the tables above. We are also indebted to Dr. F. D. Chattaway, F.R.S., for a supply of picene, to the Dyestuffs Group of Imperial Chemical Industries for several gifts of material, and to Professor G. T. Morgan, F.R.S., for a sample of perylene. We wish also to express our thanks to all our laboratory assistants, whose good work has in various ways helped these investigations.

Summary.

Tests for cancer-producing action on mice are in progress, or have been completed, with preparations of the following polycyclic aromatic hydrocarbons, composed entirely of benzene rings :—(1) All the six possible 4-ring compounds, (2) all the ten known compounds out of the fifteen possible 5-ring compounds, (3) some compounds containing six and eight rings, and others. Some of the hydrocarbons examined are of very low solubility, and all tests carried out with them are hence unsatisfactory. At the present stage of the investigation, no hydrocarbons in the pure state have produced cancers except 1 : 2 : 5 : 6-dibenzanthracene and some compounds closely related to it. Some impure specimens of other hydrocarbons (*e.g.*, 1 : 2 : 3 : 4-dibenzanthracene) have produced tumours, but the substances to which these effects are due have not been identified. 1 : 2 : 5 : 6-dibenzanthracene shows undiminished carcinogenic power when very highly purified ; it has been shown to be active in nine different media, and has produced cancer of the skin when applied in a concentration of 0.003 per cent. in benzene. Methods for the purification of the hydrocarbon are described. Two hundred and thirty-three mice treated with solutions of this compound in benzene yielded 77 epitheliomas and 21 papillomas. The 3'-methyl derivative of 1 : 2 : 5 : 6-dibenzanthracene is less active. The carcinogenic power of some closely allied compounds is described in the following paper.

Table VI.—6-Ring and 8-Ring Compounds.

	Solution.			Mice.			Tumours.	
	Solvent.	Concen- tration per cent.	Saturated	Initial number.	Alive after—		Epi- thelioma.	Papil- loma.
					6 months.	1 year.		
6-Ring Compounds.								
 2' : 1'-anthra-1 : 2-anthracene	Xylene	?	+	10	5	None	0	0
	Tetralin	0.2	+	20	5	1	0	0
	Benzene	0.25	+	5	4	3	0	0
	(1) Prepared by Clar's method (Clar, Wallenstein and Avenarius, 1929), m.p. 389° (2) From Dr. Clar							
 1' : 2'-anthra-1 : 2-anthracene	Benzene	0.1	+	10	3	2	0	0
	Xylene	?	+	10	None	None	0	0
	Tetralin	0.2	+	10	1	None	0	0
	Benzene	0.25	+	5	4	3	0	0
(1) Prepared by Clar's method (<i>loc. cit.</i>), m.p., in sealed tube, 304° (2) From Dr. Clar								
1 alive, day 685, negative,								

DESCRIPTION OF PLATES.

PLATE 15.

FIG. 20.—Mouse 51/32. Primary tumour. 1:2:5:6-Dibenzanthracene purified by H_2SO_4 and then by passage through picrate. 0.003 per cent. in benzene. 702nd day. $\times 110$.

PLATE 16.

FIG. 21.—Mouse 58/31, Series 11. Primary tumour. 1:2:5:6-Dibenzanthracene purified by H_2SO_4 and then by passage through picrate. 336th day. Spindle-celled (left) and squamous-celled (right) types. $\times 30$.

FIG. 22.—Mouse 49/31, Series 9. Primary tumour. 1:2:5:6-Dibenzanthracene purified by passage through picrate and then by H_2SO_4 . 335th day. Note permeation of tumour through sub-epidermal tissue. $\times 40$.

PLATE 17.

FIG. 23.—Same mouse as Fig. 22. Invasion of a nerve in subcutaneous tissue by epithelioma. $\times 110$.

FIG. 24.—Same mouse as Fig. 22. Metastasis in axillary gland. $\times 55$.

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FIG. 20.

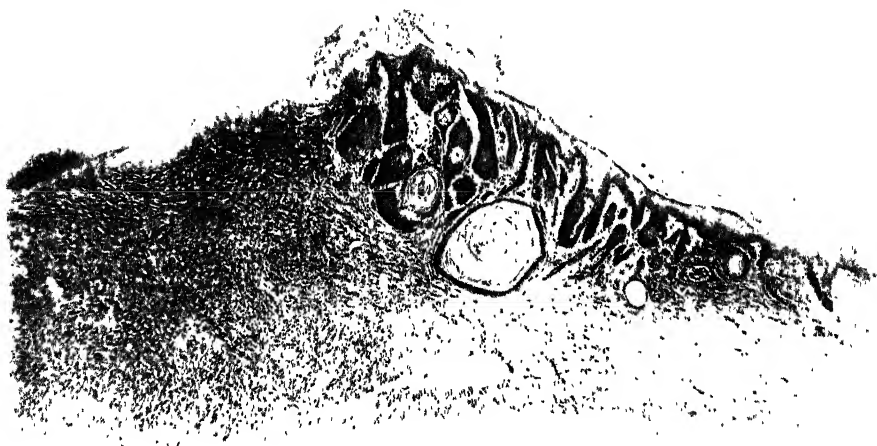


FIG. 21.



FIG. 22.

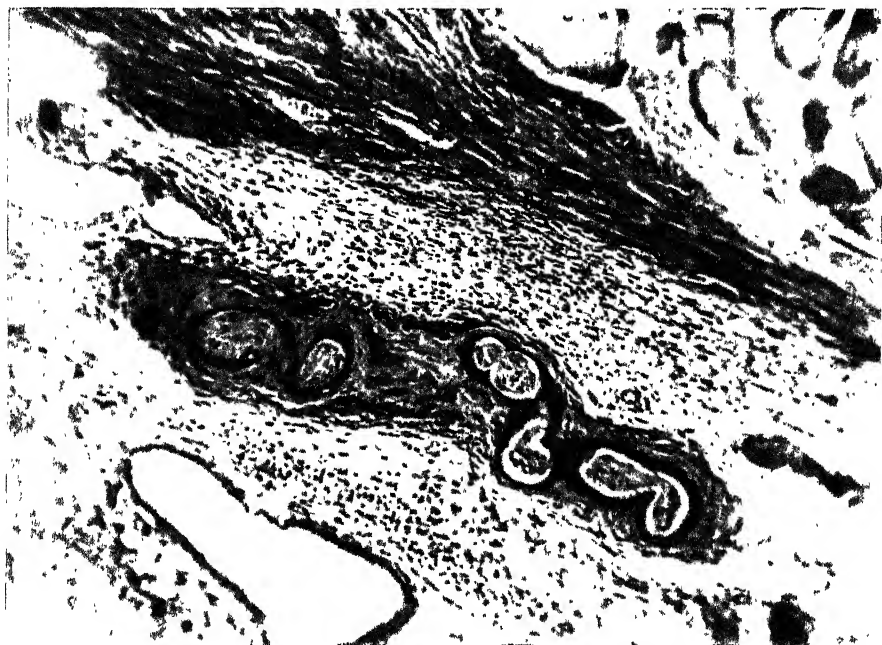


FIG. 23.

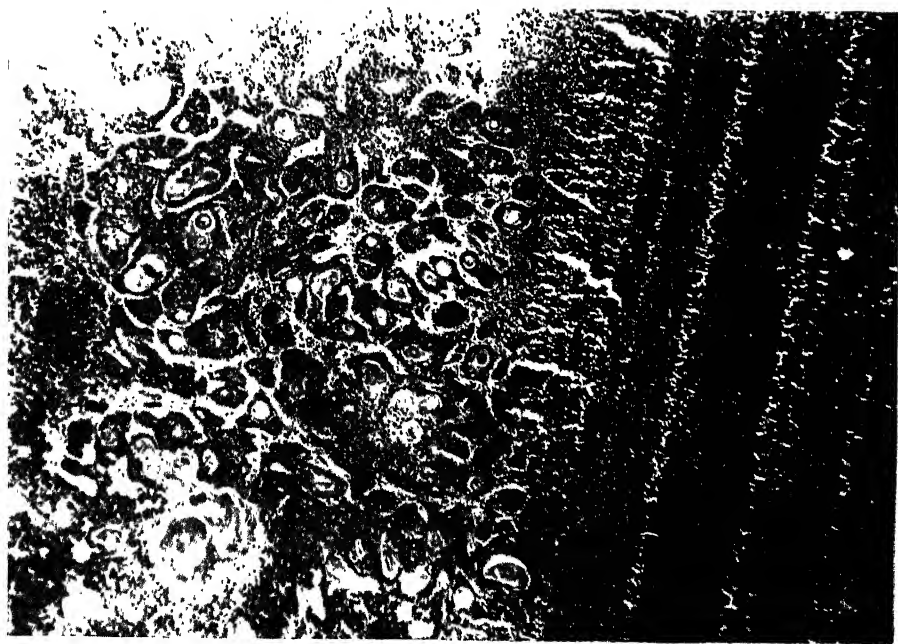


FIG. 24.

The Production of Cancer by Pure Hydrocarbons.—Part II.

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(From the Research Institute, The Cancer Hospital (Free), London.)

(Communicated by Sir Henry Dale, Sec.R.S.—Received May 12, 1932.)

[PLATES 18-21.]

The experiments described in this and the preceding communications form part of a systematic search for cancer-producing activity among the whole group of polycyclic aromatic hydrocarbons. In selecting for study the compounds to be discussed in the present communication, attention has been directed mainly to three considerations:—

- (a) A pure hydrocarbon of known chemical structure which should have the same fluorescence spectrum as carcinogenic mixtures of unknown composition has been sought.
- (b) The rapid appearance of tumours in mice upon application of certain carcinogenic tars and mixtures affords some evidence for the existence of pure compounds of more powerful carcinogenic activity than 1:2:5:6-dibenzanthracene, and the production of such substances has been attempted.
- (c) The chief object has been to ascertain the molecular conditions which are necessary for the development of carcinogenic activity, and to establish a relationship between cancer-producing power and chemical constitution.

Reference to (a) has already been made in Part I (p. 456).

Examination of members of the benzantracene group of hydrocarbons showed that while 1:2-benzanthracene and 6-methyl-1:2-benzanthracene had little, if any, carcinogenic activity, there was evidence that such activity was developed to a low degree by the introduction of the isopropyl group at position 6. One hoped, therefore, that introduction of a suitable substituent into a suitable position in the 1:2:5:6-dibenzanthracene molecule would lead to enhanced carcinogenic activity. This hope has not yet been realised, for every derivative of 1:2:5:6-dibenzanthracene so far examined has shown less carcinogenic power than the parent substance. A considerable number of new derivatives have been studied, but it has not yet been possible

to cover more than a small part of the very wide field which is opened by varying the nature and position of the substituents, and it is by no means certain that substitution invariably depresses the cancer-producing activity of 1:2:5:6-dibenzanthracene. Investigations along these lines are being continued.

The problem of correlating cancer-producing activity with chemical constitution is one which can only be solved by examination of an exceedingly large number of new compounds, and it would be premature to generalise at the present stage. At the same time, a number of somewhat significant facts have emerged, and it will be well to direct attention to the general trend of these, without claiming any finality.

Hydrocarbons Derived from 1:2-Benzanthracene. (Table I.)

As already stated, there are indications that the 1:2-benzanthracene molecule is rendered carcinogenic by the introduction of the *isopropyl* group at

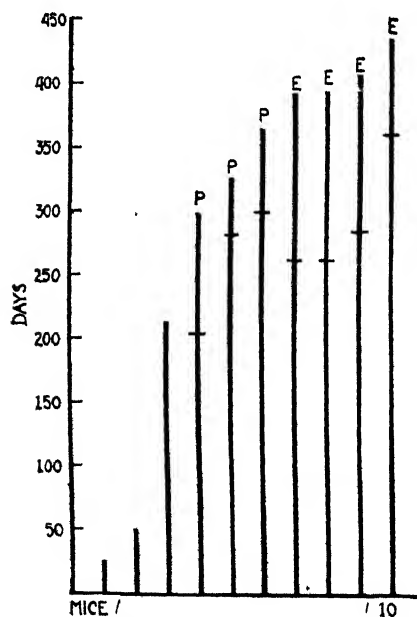


FIG. 1.—Mixture containing 6-isopropyl-1:2-benzanthracene.*

position 6. A mixture containing 6-*isopropyl* 1:2-benzanthracene, applied in 0.3 per cent. solution in benzene, produced 7 tumours (1 non-malignant, 2 doubtful and 4 definite cancers) in a series of 10 mice during 13 months, fig. 1 and fig. 4, Plate 18.

The sample of 6-*isopropyl*-1:2-benzanthracene which gave positive results was not free from contamination with some related compounds, so that confirmation of these results is being sought by tests which are in progress on a chemically pure specimen of 6-*isopropyl*-1:2-benzanthracene. In the meantime, it suffices to say that the derivatives of 1:2-benzanthracene now under test have been selected as preliminary material for a comprehensive survey of homologues of 1:2-benzanthracene,

from which it should be possible to define precisely the conditions of substitution which are necessary for carcinogenic power.

* For explanation of these charts, figs. 1 to 3, see Part I, p. 466.

5 : 6-cycloPenteno-1 : 2-benzanthracene (Formula III). (Table II.)

Positive results have been obtained with 5 : 6-cyclopenteno-1 : 2-benzanthracene, a compound which occupies an intermediate position between a 6-alkyl derivative of benzanthrane and 1 : 2 : 5 : 6-dibenzanthracene. Moreover, the graph given in Part I (p. 467) appears to indicate that this *cyclopenteno* compound is superior in carcinogenic power to 1 : 2 : 5 : 6-dibenzanthracene, for, with the compound now under consideration, on the whole tumours appear more rapidly. Table II shows that a total of 23 tumours (18 epitheliomas and 5 papillomas) were obtained in 50 mice, of which 26 were alive after six months. The fact that 5 : 6-cyclopenteno-1 : 2-benzanthracene so closely resembles 1 : 2 : 5 : 6-dibenzanthracene in its carcinogenic power is of interest, not only on account of the structural resemblance between the two compounds, but also on account of their differences, for, chemically, the partially reduced five-membered ring of the *cyclopenteno* compound bears little resemblance to the similarly situated benzene ring of the dibenzanthracene.

The tumours produced by 5 : 6-cyclopenteno-1 : 2-benzanthracene show abundant invasion of voluntary muscle, and present all the usual appearances of malignant tumours of the skin in mice.* Metastases have been more numerous in these mice than in any others used in the work on carcinogenesis which has been carried out in this Institute. Five of the mice painted with the purest specimens of the hydrocarbon showed metastases as follows :—

				Site of metastases.
Series 4	Mouse 1	.. Glands of both axillæ.
			„ 2	.. Lung, fig. 8, Plate 20.
Series 5	Mouse 1	.. Axillary gland.
			„ 2	.. Axillary gland and lung.
			„ 3	.. Lung, fig. 7, Plate 19.

A comparison between the data given in fig. 2 of the preceding paper (p. 467) and those summarised in the following table shows that the tumours produced by 5 : 6-cyclopenteno-1 : 2-benzanthracene appear on the whole earlier than do

* In a subsequent paper, experiments carried out in conjunction with Miss Geraldine Barry, F.R.C.S., will be described, in which spindle-celled tumours of the connective tissue of mice have been produced by subcutaneous injection of this compound in a fatty medium.

those due to 1:2:5:6-dibenzanthracene or its 3'-methyl derivative, since about one-half appear before the 185th day.

Time of Appearance of 23 Tumours of the Skin produced by
5:6-*cyclo*Penteno-1:2-benzanthracene.

Day.		Number of tumours.	Per cent.
143 to 184 (41 days)	12	52.2
185 to 285 (100 days)	9	39.1
286 to 305 (19 days)	2	8.7
		—	—
		23	100.0
		—	—

The incidence of tumours in Series 3 and 5 is illustrated by the graphs, figs. 2 and 3, and photographs of the tumours are given in figs. 5 to 8, Plates 18 to 20.

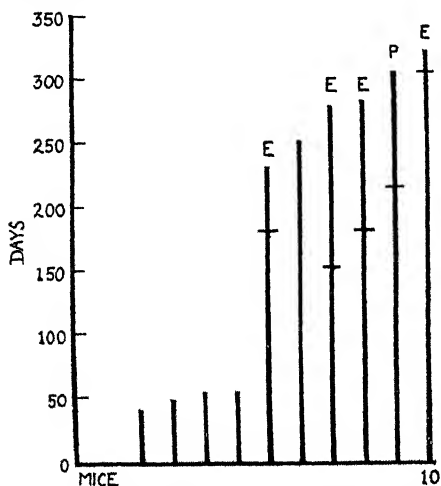


FIG. 2.—5:6-*cyclo*Penteno-1:2-benzanthracene (Series 3).

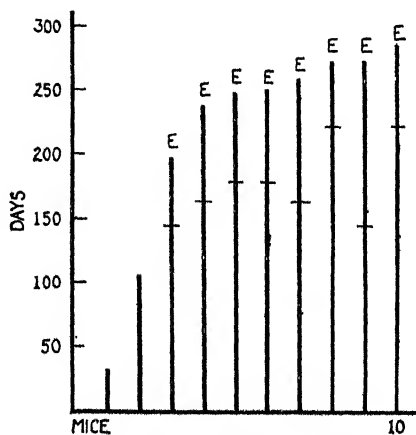
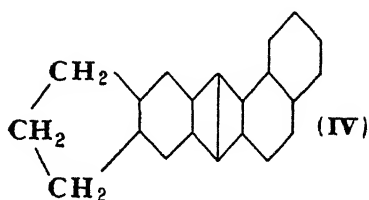
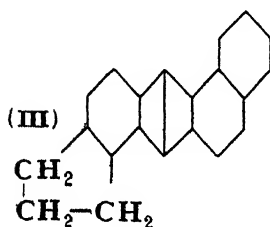
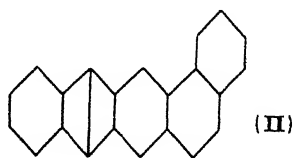
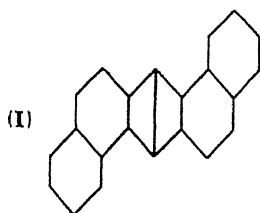


FIG. 3.—5:6-*cyclo*Penteno-1:2-benzanthracene (purified through picrate) (Series 5).

A factor common to 6-*isopropyl*-1:2-benzanthracene, 5:6-*cyclopenteno*-1:2-benzanthracene (Formula III) and 1:2:5:6-dibenzanthracene (Formula I) is that all three compounds are benzanthracene derivatives substituted at position 6. From this standpoint the behaviour of compounds in which a fifth ring is condensed in the 6:7-position instead of the 5:6-position is of importance. One such compound, 2':3'-naphtha-2:3-phenanthrene

(Formula II), has been dealt with in Part I, and has given negative results. This, however, is hardly a fair subject for comparison, because chemical evidence shows that the "reactive" ring of the anthracene system, represented in the conventional way by the "bridge" bond (indicated by the vertical line within the hexagon), is not the central ring of the molecule, as is the case with 1:2:5:6-dibenzanthracene.



A more suitable test case is afforded by 6:7-cyclopenteno-1:2-benzanthracene (Formula IV). This substance is still under examination, but it may be stated that its carcinogenic activity appears at present to be less than that of the 5:6-cyclopenteno compound (two papillomas in eight mice surviving out of ten after nine months—*cf.* figs. 2 and 3). Therefore it seems that the most favourable type of molecular structure for carcinogenic activity is attained by the attachment of new rings to the 1:2- and 5:6- positions of the anthracene complex. It may well be that simpler anthracene derivatives with non-cyclic substituents at 1, 2, 5 and 6 will be carcinogenic, and it is proposed to examine this possibility. As a preliminary step, 1:5-di-isopropenylantracene has been tested, with negative results.

Compounds Derived from 1:2:5:6-Dibenzanthracene. (Table III.)

For reasons already explained, a number of simple derivatives of 1:2:5:6-dibenzanthracene have been tested for carcinogenic activity. The diminished carcinogenic power of the 3'-methyl derivative has been dealt with in Part I.

The 2'-methyl compound has produced five tumours, all of them malignant, in 20 mice. This compound appears then, so far as one can draw any conclusions at all from small numbers, to be intermediate in carcinogenic power between the parent hydrocarbon 1:2:5:6-dibenzanthracene (98 tumours in 233 mice), and the 3'-methyl compound (21 tumours in 120 mice). Thus the ratios of these pairs of numbers are approximately 1:4, 1:2.3, and 1:6. One of the tumours produced by the 2'-methyl compound is of especial interest, in that the primary tumour was chiefly spindle-celled in structure, while the metastases which were present in the glands of both axillæ were squamous-celled, figs. 9-11,* Plates 20 and 21.

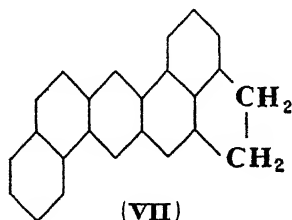
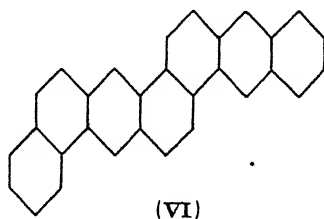
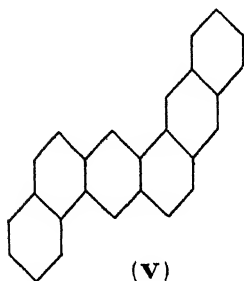
The 9:10-dialkyl derivatives which have been examined also show reduced activity in comparison with the parent compound. The 9:10-dibenzyl compound has produced two epitheliomas in a series of ten mice. It seemed desirable to examine various derivatives containing oxygen and nitrogen. Several such compounds were applied to mice, and of these, 9-methoxy-1:2:5:6-dibenzanthracene has produced one epithelioma, and two out of five mice living which have been painted with the 9-amino compound bear tumours. On the whole, then, substitution, particularly in the *meso* positions, seems to lessen the carcinogenic power of the 1:2:5:6-dibenzanthracene molecule. It is also noteworthy that reduction very largely destroys the carcinogenic properties of 1:2:5:6-dibenzanthracene, since the octahydro-derivative has given only one papilloma. In many of these series a considerable proportion of the mice are still alive, so that no final statement about the inactivity of these compounds can as yet be made.

Effect of Increasing the Molecular Complexity of 1:2:5:6-Dibenzanthracene.
(Table IV.)

The addition of another ring to the 1:2-benzanthracene molecule having resulted in a change from an inactive substance to substances strongly carcinogenic, it was natural to enquire into the effect of adding extra rings to the 1:2:5:6-dibenzanthracene molecule. Two such cases have been mentioned in Part I, namely, 1:2:3:4:5:6-tribenzanthracene and 4:5:10:11-di-(1':2'-naphtha)chrysene. In addition, 2':3'-phenanthra-1:2-anthracene (Formula V) has given one papilloma in 10 mice, and 4:5-benz-10:11-(1':2'-

* A beautiful example of this difference in structure between primary growth and metastases was demonstrated recently, in specimens from a tar-painted mouse, by Dr. J. A. Murray, F.R.S., before the Pathological Section of the Royal Society of Medicine.

naphtha) chrysene (Formula VI) has so far given only negative results; the seven-ring compound was applied in tetralin solution as well as in benzene. It is thus evident that the molecular complexity must be restricted within fairly narrow limits for the manifestation of cancer-producing activity.



Positive results were obtained with phenanthra-acenaphthene (Formula VII), in which an additional five-membered ring is condensed with the 1:2:5:6-dibenzanthracene molecule. This may, however, be more properly regarded as a dialkyl derivative of 1:2:5:6-dibenzanthracene. A pure preparation of this compound has given one epithelioma in a series of five mice only, and one of these, which is still alive, bears a well-developed tumour.

Miscellaneous Compounds.

In addition to the hydrocarbons already mentioned, a few of miscellaneous type have been tested for carcinogenic activity, with negative results. These are given in Table IV, and do not call for special notice. Acenaphthylene and its polymeride were examined on account of their probable presence in coal-tar. The constitution of the saturated dimeride of 1-isopropenylnaphthalene is unknown; it may possibly be a derivative of 1:2:5:6-dibenzanthracene. Finally, 3:4-benzphenanthrene, which is still under test, is the last remaining hydrocarbon composed of four benzene rings (compare Part I, p. 461).

The synthetic work involved in the production of the substances referred to in this communication has been described in a series of papers on Polycyclic Aromatic Hydrocarbons.*

The author is very much indebted to Professor E. L. Kennaway, who has been responsible for the experiments on animals described in this communication.

* 'J. Chem. Soc.,' p. 1087 (1930); pp. 487, 489, 499, 2012, 2524, 2529, 3273 (1931); p. 456 (1932).

Table I.—Derivatives of 1:2-Benzanthracene.

Compound.	Per cent. in benzene.	Mice.			Day of death of last mouse.	Duration of experi- ment on 1.5.32.	Tumours.	
		Initial number.	Alive after				Epi- thelioma.	Papil- loma.
			6 months.	1 year.				

2'-Methyl-1:2-benzanthracene	0.3	10	6		5 alive	318		
3-Methyl-1:2-benzanthracene	0.3	10	3		3 alive	316	0	0
6-Methyl-1:2-benzanthracene	0.25-0.3	14	9	4	458			
7-Methyl-1:2-benzanthracene	0.3	10	8		7 alive	306		
2'-6-Dimethyl-1:2-benzanthracene	0.3	10	5		5 alive	189		
2'-7-Dimethyl-1:2-benzanthracene	0.3*	10	7		6 alive	279		
3'-6-Dimethyl-1:2-benzanthracene	0.3	10	8		9 alive	259		
3'-7-Dimethyl-1:2-benzanthracene	0.3	10	9		10 alive	196		
6:7-Dimethyl-1:2-benzanthracene	0.3	10	7		10 alive	162		
3-isoPropyl-1:2-benzanthracene	0.3	10			7 alive	290		
6-isoPropyl-1:2-benzanthracene—								
(1) Crude mixture, m.p. 114°-115°	0.3	10	8	4	435		4	3
(2) Pure, m.p. 131°-132°	0.3	10			9 alive	177		
(3) Purified through quinone, m.p. 132°-133°	0.3	7			6 alive	157		
7-isoPropyl-1:2-benzanthracene	0.3	10			9 alive	173		
10-isoPropyl-1:2-benzanthracene	0.3	10	6	3	3 alive	406		
6-Phenyl-1:2-benzanthracene	0.25	20	12	6	651		0	0
9:10-Diphenyl-1:2-benzanthracene	0.25	20	11	7	652		0	0
10-Benzyl-1:2-benzanthracene	1.0, 0.5	10	8	3	466		0	0
1:2:1':2'-Dibenz-6:6' (or 7:7'-dianthryl)	0.3*	10	7	0	315		0	0

* With the exception of these two, none of the solutions was saturated.

Table II.—5 : 6-*cyclo*Penteno-1 : 2-benzanthracene.

Series.	Sample.	Per cent. in benzene.	Mice.			Day of death of last mouse.	Tumours.	
			Initial number.	Alive after			Epithelioma.	Papilloma.
				1 year.				
				6 months.				
1	Mixture of isomers, m.p. 142°-168°	0.3	10	4	0	358	1	2
2	Pure, m.p. 197°-199° Purified through picrate	0.3	10	3	0	224	1	2
3		0.3	10	5	0	321	4	1
4		0.1	10	6	0	339	4	0
5		0.3	10	8	0	285	8	0
			50	26			18	5

Table III.—Derivatives of 1 : 2 : 5 : 6-Dibenzanthracene.

Compound.	Solution.		Mice.			Day of death of last mouse.	Duration of experiment on 1.5.32.	Tumours.	
	Per cent. in benzene.	Saturated.	Initial number.	Alive after				Epi-thelioma.	Papil-loma.
				6 months.	1 year.				
2'-Methyl-1 : 2 : 5 : 6-dibenzanthracene	0.3	+	20	14	12	730	—	5	0
9 : 10-Dimethyl-1 : 2 : 5 : 6-dibenzanthracene .	0.3	+	5	3	1	410	—	0	1
9 : 10-Di- <i>n</i> -butyl-1 : 2 : 5 : 6-dibenzanthracene	0.3	—	10	8	1	1 alive	631	—	—
9 : 10-Dibenzyl-1 : 2 : 5 : 6-dibenzanthracene...	0.3	—	10	5	3	450	—	2	0
9 : 10-Dibenzyl-1 : 2 : 5 : 6-dibenzanthracene (crude sample)	0.3	—	10	8	4	561	—	1	0
9-Acetoxy-1 : 2 : 5 : 6-dibenzanthracene.....	0.3	+	10	9	5	4 alive	465	—	—
9-Methoxy-1 : 2 : 5 : 6-dibenzanthracene	0.3	—	10	0	—	180	—	—	—
.....	0.3	—	10	3	0	298	—	1	—
.....	0.3	—	20	0	—	18 alive	95	—	—
1 : 2 : 5 : 6-Dibenz-9-anthranol (impure).....	0.3	+	10	0	—	129	—	—	—
.....	0.3	+	10	5	—	4 alive	319	—	—
.....	0.3	+	10	8	—	8 alive	291	—	—
.....	0.3	+	10	0	—	88	—	—	—
.....	0.3	+	10	6	—	6 alive	286	—	—
9-Nitro-1 : 2 : 5 : 6-dibenzanthracene	0.3	+	10	4	4	3 alive	438	—	—
9-Amino-1 : 2 : 5 : 6-dibenzanthracene	0.3	+	10	7	—	5 alive	368	—	—
9-Diacetylamino-1 : 2 : 5 : 6-dibenzanthracene	0.3	+	10	0	—	175	—	0	—
9- <i>n</i> -Butyrylamino-1 : 2 : 5 : 6-dibenzanthracene	0.3	+	10	3	—	3 alive	320	—	—
<i>N</i> -1 : 2 : 5 : 6-Dibenzanthranylsuccinimide.....	0.3	+	10	5	—	3 alive	291	—	—
Octahydro-1 : 2 : 5 : 6-dibenzanthracene	0.3	—	10	7	2	431	—	0	1
<i>cis</i> -9 : 10-Dimethyl-9 : 10-dihydro-1 : 2 : 5 : 6-dibenzanthracene	0.3	—	10	6	3	538	—	0	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	—
.....	0.3	—	10	—	—	—	—	—	

* Two of the mice still living bear tumours.

Table IV.—Miscellaneous Compounds.

Compound.	Solution.			Mice.			Day of death of last mouse.	Dura- tion of experi- ment on 1.5.32.	Tumours.	
	Solvent.	Concen- tration per cent.	Satu- rated.	Initial number.	Alive after				Epithel- ioma.	Papil- loma.
					1 year.					
					6 months.					
Acenaphthylene.....	Benzene	0.25	—	20	13	7	522	0	0	
Polycenaphthylene [(C ₁₂ H ₈) ₁₂]	"	2.0	—	10	6	0	308	0	0	
Dimeride of 1-isopropenylnaphthalene (C ₂₀ H ₂₄)	"	0.3	—	10	1	1	1 alive	413		
"	"	0.3	—	10	10		10 alive	296		
1-Phenylanthracene	"	0.5-0.3	0.3	10	10	7	768†		0	
2-Phenylanthracene	"	0.5	—	10	8	4	720		0	
9:10-Dibenzylanthracene	"	0.5, 0.3	—	10	7	5	628§		0	
1:5-Di-isopropenylnanthracene	"	0.3	—	10	2		1 alive	288		
3:4-Benzphenanthrene	"	0.3	—	20	18		18 alive	261		
Acenaphthanthracene	"	0.5	—	20	6	2	437	0	0	
10-Benzylacenaphthanthracene	"	1.0, 0.5	0.5	20	7	3	710	0	0	
Phenanthra-acenaphthene (Formula VII) (crude)	"	0.3	—	10	3	1	425	2	0	
"	"	0.3	—	10	5				*	
"	"	0.3	—	10	10				*	
"	"	0.3	—	5	3	2	5 alive	305		
"	"	0.3	—	5	3		9 alive	283		
"	"	0.3	—	5	3		2 alive	512		
"	"	0.3	—	5	3		3 alive	252		
"	"	0.3	—	10	9	4	9 alive	206		
2':3'-Phenanthra-1:2-anthracene (Formula V).....	"	0.16	+ 40°	10	7	2	2 alive	620	*	
2':3'-Phenanthra-2:3-phenanthrene	"	0.25	+ 40°	5	4	3	651	459	0	
"	Xylene	0.17	+ 40°	5	5	3	1 alive	228	0	
Phenanthrafluorene	Benzene	0.3	+	10	9	0	214	0	0	
"	"	0.3	+	10	2	0			0	
"	"	0.3	—	5	4	1	1 alive	439	†	
1:2-Diphenyl chrysene	"	0.2	+	10	5	3	573	0	0	
4:5-Benz-10:11-(1':2'-naphtha)chrysene (For- mula VI)	"	0.2	+	10	1	0	184	0	0	
4:5-Benz-10:11-(1':2'-naphtha)chrysene	Tetralin	0.2	+	20	15	7	435	0	0	
"	"									

* One mouse still living bears a distinct tumour.

† One mouse still living bears a doubtful tumour.

‡ No more material available after day 531.

§ No more material available after day 476.

Summary.

Preliminary results suggest that 6-isopropyl-1:2-benzanthracene is carcinogenic, and a pure sample of this has been synthesised and is being examined for carcinogenic activity, together with other isopropyl and methyl derivatives of 1:2-benzanthracene. Of compounds containing the 1:2:5:6-dibenzanthracene ring system, the following have produced tumours: the 2'-methyl, 3'-methyl, 9-amino, 9-methoxy, and 9:10-dibenzyl derivatives of 1:2:5:6-dibenzanthracene, and phenanthra-acenaphthene. In all cases the activity was less than that of 1:2:5:6-dibenzanthracene, but the allied compound 5:6-cyclopenteno-1:2-benzanthracene proved more active than any of these. Metastases in the axillary glands and lungs were obtained in five mice to which this cyclopenteno compound was applied. There is evidence that a molecular structure consisting of new rings attached to the 1:2- and 5:6- positions of the anthracene ring system is particularly efficacious in promoting carcinogenic activity.

[*Note added in proof (August 16th, 1932).*—Since this paper was written confirmation of the carcinogenic activity of 6-isopropyl-1:2-benzanthracene has been obtained. For the pure sample, m.p. 131-132° (see Table I) has given six tumours in ten mice; furthermore, six out of the seven mice treated with the highly purified specimen, m.p. 132-133°, have developed tumours.]

DESCRIPTION OF PLATES.

PLATE 18.

FIG. 4.—Mouse 96/31. Mixture containing 6-isopropyl-1:2-benzanthracene. Primary tumour. Note invasion of muscle. Day 435. $\times 40$.

FIG. 5.—Mouse 55/32. 5:6-cyclopenteno-1:2-benzanthracene purified by passage through picrate. Day 285.

PLATE 19.

FIG. 6.—Same mouse as Fig. 5. Primary tumour. Day 285. $\times 37$.

FIG. 7.—Same mouse as Fig. 5. Metastasis in lung. $\times 55$.

PLATE 20.

FIG. 8.—Mouse 29/32. 5:6-cyclopenteno-1:2-benzanthracene, 0.1 per cent. in benzene. Metastasis in lung. Day 281. $\times 45$.

FIG. 9.—Mouse 110/31. Primary tumour. 2'-Methyl-1:2:5:6-dibenzanthracene. 414th day. Note spindle-celled structure and invasion of voluntary muscle. $\times 55$.

PLATE 21.

FIGS. 10 and 11.—Same mouse as Fig. 9. Metastases in right (fig. 10) and left (fig. 11) axillæ. $\times 55$.



FIG. 4.



FIG. 5.

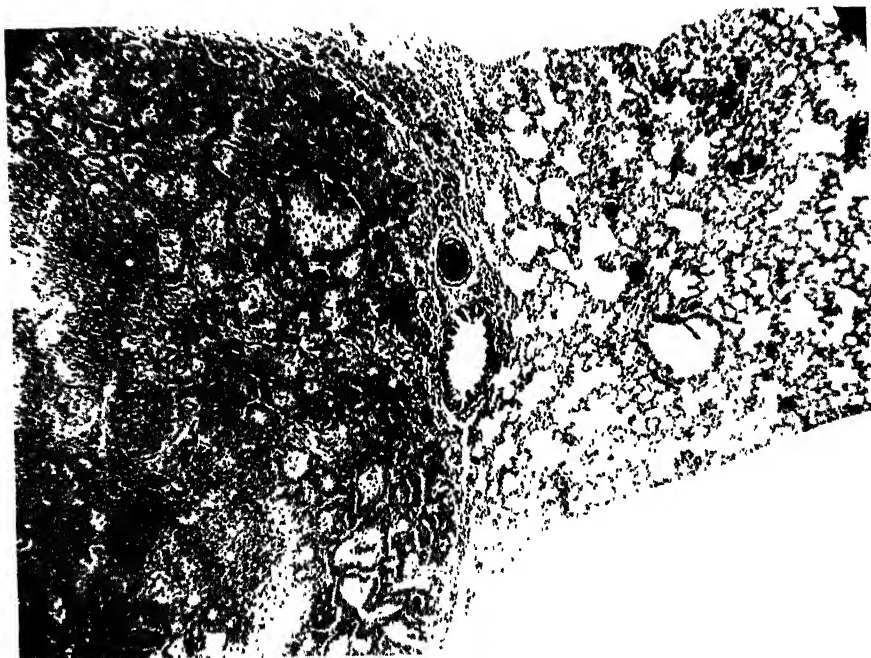


FIG. 7.

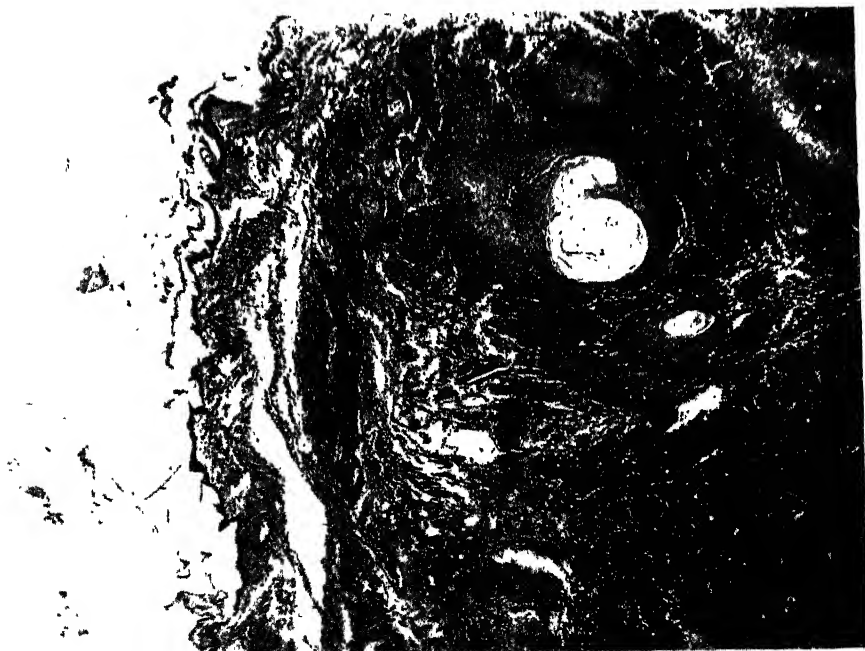


FIG. 6.



FIG. 8.

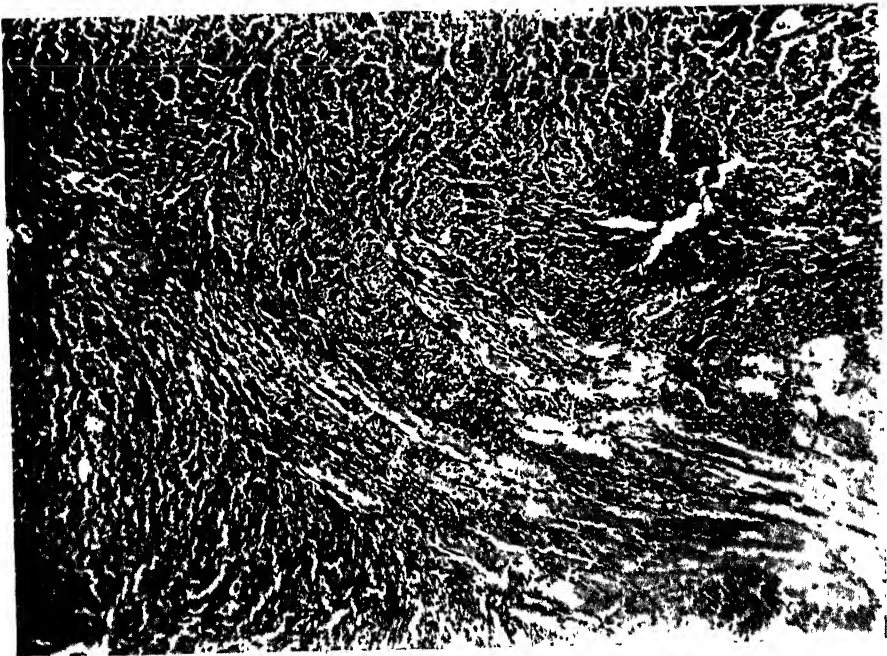


FIG. 9.

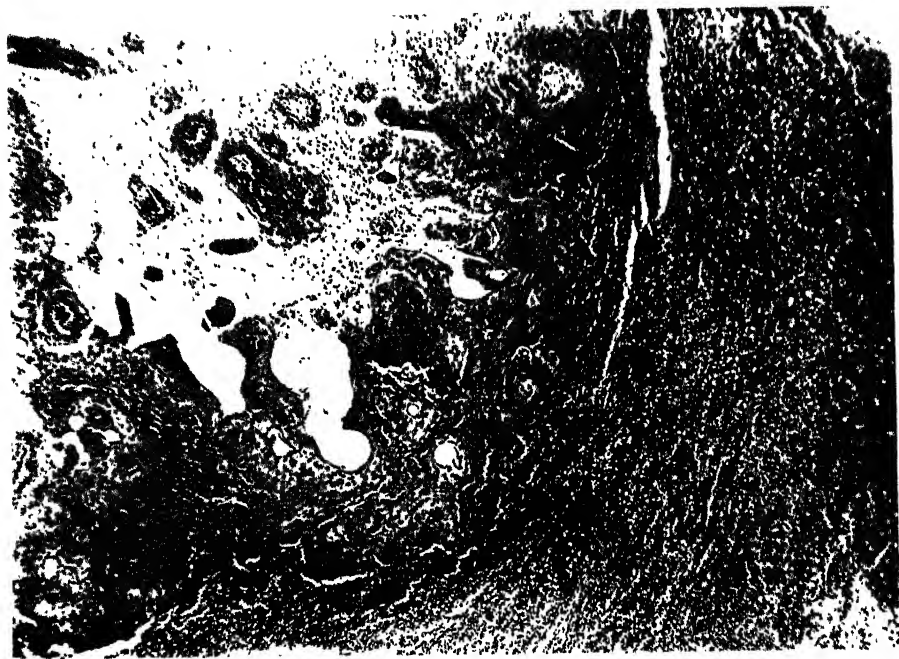


FIG. 10.

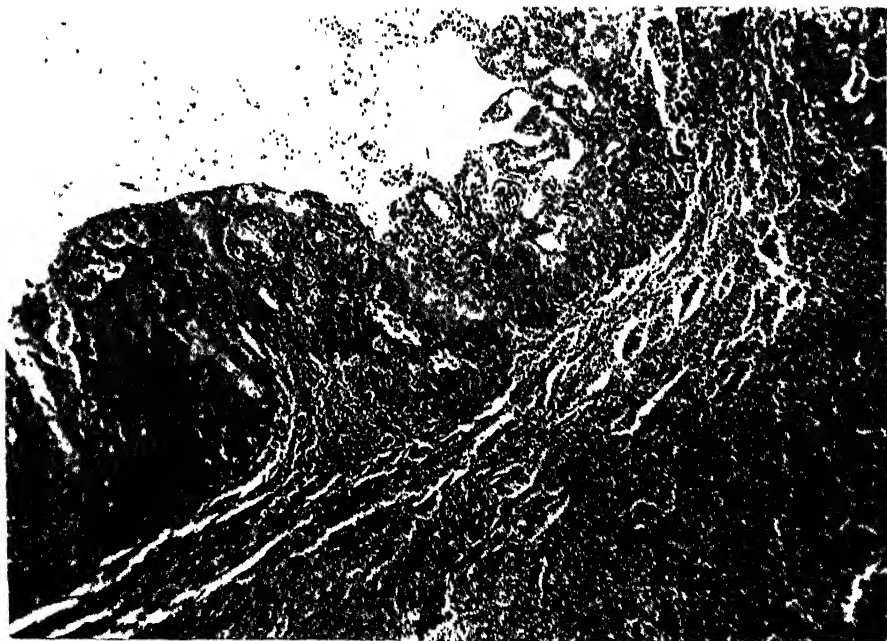


FIG. 11.

The Development in vitro of the Blood of the early Chick Embryo.

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(Communicated by J. T. Wilson, F.R.S.—Received June 9, 1932.)

[PLATES 22-25.]

Introduction.

In a series of tissue cultures of fragments taken from different parts of young chick embryos, at various stages, it was found that differentiation frequently occurred, the characteristic cells of highly specialised tissues appearing in cultures in which they could not possibly have been present at explantation. The most striking differentiations were the development of red blood corpuscles, capillary vessels, nerve cells with axons and of rhythmically contracting cardiac muscle.

This paper describes a simple method by which may be obtained regularly and quickly the development of very large numbers of red cells, and an account is given of the histology of the cultures. It is intended to be introductory to a physiological study, now in progress, of the conditions of hæmatopoiesis.

Literature. Erythropoiesis *in vitro* has been reported by several authors, but it has not been thoroughly investigated and, with the exceptions of the works of Slonimski (1930, *a*, 1931) and Shipley (1915-16), the earlier papers have concerned the somewhat sporadic appearance of small numbers of erythrocytes. In the earlier works, also, the explants were derived from hæmatopoietic organs, or from that part of the embryo in which blood would normally have developed at latest quite soon after the time at which the experiment was made. The present paper, on the other hand, is based principally upon cultures of fragments of the primitive streak—that is, upon explants of presumptively hæmatopoietic cells isolated before they had arrived at the normal hæmatopoietic region of the embryo. The literature may be very briefly summarised as follows: Shipley (1915-16) made plasma cultures from the *area opaca* of chick embryos at a time prior to the formation of the blood islands, and obtained the differentiation of erythrocytes from amoeboid cells. Erythropoiesis is reported by N. G. and A. L. Chlopin (1925) in cultures of Axolotl spleen; by Erdmann, Eisner, and Laser (1925-26), in cultures of embryonal rat spleen; by Freifeld and Ginsburg (1927) in cultures of rabbit adrenals; by de Haan (1928-29) in

cultures of blood cells of the horse; by Timofejewsky and Benewolenskaja (1929) in cultures of blood from a case of acute myeloid leukaemia, and by Benewolenskaja (1930) in cultures of embryonal human liver. Slonimski (1930 *a*, 1931), using *Rana fusca* and Axolotl embryos, excised the blood island zone at early stages, and kept it as a culture enclosed in a sheath of epiblastic epithelium. The little cyst became full of red blood in an abundant plasma, and there were vessels with endothelial walls.

Nomenclature.—In her study of the early stages of hæmatopoiesis in the *area vasculosa* of the living chick embryo, Sabin (1920) rightly distinguishes between the blood islands and the “angioblasts,” pointing out that the one term “blood island” should not be used for these two different structures. The “angioblasts” are masses of cells or single cells formed from the mesenchyme, from which develop the endothelial vessels containing plasma and the blood islands; the blood islands proper are the groups of cells or single cells derived directly from the “angioblasts,” which persist attached to the endothelial walls, and whose component cells, when separated from one another by the dissolution of the blood islands, become the primitive blood cells. Such a distinction is justified, for “angioblasts” and blood islands are two distinct developmental stages differing from one another in prospective significance and in structural relationships. To “angioblast,” however, I prefer the term “hæmangioblast.” This expresses the fact that both endothelium and blood develop from the solid mass, whereas the term “angioblast” strictly refers only to the vessels, *i.e.*, to the endothelium. To avoid ambiguity, to accord with His’s use of the term “angioblast” to mean a layer of cells rather than individual cells, and in order to avoid the clumsy phrases “hæmangioblastic mass” and “mass of hæmangioblasts,” I restrict the term “hæmangioblast” to the mass and use for the component cells the infrequently needed term “hæmangioblast cell.”

MATERIAL AND METHODS.

Material.—All the cultures with which this paper is concerned were derived from embryos having a primitive streak. The stages varied from that of the early, pear-shaped *area pellucida* with a well-developed primitive streak, but no head-process, to that at which there was an advanced head process, and a head fold but no somites. The great majority of the explants were posterior halves of primitive streaks of embryos taken shortly before or soon after the appearance of the head process.

Eggs incubated for periods likely to provide embryos at suitable stages (the time varied greatly from egg to egg and in different seasons) were opened, the albumen was poured off, and the yolks decanted into petrie dishes containing 0.75 per cent. saline. The blastoderm was then dissected off, cleaned of yolk, and transferred to some smaller vessel, usually a watch glass. The part which it was intended to cultivate was cut out of the embryo with needles sharpened into knives.

Media and Method of Cultivation.—Two different media were used, one fluid and the other coagulated. For the study of the later stages of erythropoiesis, if material were required for sectioning, and for experiments intended to delimit the regions in the embryo occupied by hæmatopoietic cells, I used plasma and embryo extract. For the early stages of hæmatopoiesis, up to about the stage of dissolution of the blood islands, and if sections were not required, I used a fluid medium which was the exudate obtained by mixing plasma and extract in the proportions of two to three, allowing the mixture to clot, and cutting up the clot. This medium has the advantage of being easily removable at fixation, so not obscuring the stained specimen. In addition, it favours the formation of thinner cultures than the coagulated medium, and such cultures can more easily be stained as whole mounts. But in stages after the dissolution of the blood islands, serum and extract constitute an unsuitable medium, for there is a tendency for the cells to develop abnormally or to cease development. For this reason I have used plasma and embryo extract for all later stages. Observations based on fluid medium cultures have all been confirmed with the plasma medium.

Fluid medium cultures were made on $\frac{3}{8}$ -inch cover glasses. To obtain attachment of the usually very small explants to the cover glass the whole preparation was kept upside down during the first night, and then returned to the normal position; this ensured the attachment of all but a very few of the explants. Those which failed to become attached and remained floating became nodular, and nothing could be seen of their structure; they passed through at least the early stages of hæmatopoiesis, producing great numbers of small free young blood cells.

To study the structure of the blood islands and of their constituent cells, in whole mounts, it is essential to have blood islands which are thin enough to allow observation both during life and after staining. Primitive streak cultures usually develop one or several large blood islands containing so many cells that no structural details can be discerned. In order to obtain very small, thin blood islands, I made a large number of cultures in which the piece

of primitive streak had been cut into a number of small fragments. This did not seem to affect subsequent growth and differentiation. In spite of the extremely small size of the tiny fragments now present on each cover glass, inversion of the cultures overnight always ensured that some at least of the explants would become attached to the glass, and grow as very tiny and very thin sheets, in which the structure of the blood islands could be seen. It is interesting to note that even extremely tiny cultures, consisting of only a very few cells, were able to pass through at least the initial stages of hæmatopoiesis without abnormality.

Since dissolution of the blood islands occurred in the first two days or less, and since fluid medium cultures were not used for later stages, it was not necessary to change the medium. Plasma cultures intended for the study of later stages were sometimes transferred to fresh medium about twenty-four hours after explantation, and were not transferred again. If the change were delayed until after the dissolution of the blood islands, all or most of the cells floating in the liquefied medium were lost. In many cases I did not transfer to fresh medium at all, having found that the omission of the change did not adversely affect the development of the erythroblasts.

Histological Methods.—The material obtained in the cultures was studied in whole mounts, smears, and sections. Most of the cultures intended for whole mounts or sections were fixed in Zenker containing 10 per cent. formol, but a few, during the early part of the work, in Zenker containing 3 per cent. acetic acid. As stains I used chiefly Giemsa (Gurr and Grubler), to a lesser extent Eosin Azur II and Dominici's triple Eosin Orange G-Toluidin Blue method as modified by Dantschakoff (1908). Giemsa proved the most useful stain. Stained preparations were differentiated in absolute alcohol, and were then passed through acetone: usually through graded mixtures of absolute alcohol and acetone. Acetone differentiates very slowly, and could therefore be used for dehydration, particularly with plasma cultures in which a great thickness of relatively impenetrable plasma made rapid dehydration impossible. Preparations mounted in Gurr's neutral balsam showed, after a period of months, little or no fading.

The cells floating in the fluid after dissolution of the blood islands were studied in smears, which were dried by waving vigorously in the air and fixed rapidly in methyl alcohol. This was followed by staining in one or other of the above mixtures, and mounting in Gurr's neutral balsam. As smears, however carefully prepared, are liable to be a source of artefacts, I have carefully confirmed the results given by them with those given by cultures

fixed in Zenker-formol. Discrepancies between the results of the two methods are discussed in the descriptive section.

PART 1.

Gross Changes in the Cultures.

On the morning of the day after explantation the attachment of the cultures was complete, and outwandering had usually begun. By the evening a considerable number of cells had emigrated, and the central part of the culture consisted of a dense mass, the hæmangioblast. Each culture usually formed one large hæmangioblast, but sometimes produced several; especially was this so with cultures from parts of the embryo normally destined to be incorporated in the *area vasculosa*, but it was rare for a culture derived from the primitive streak to form more than one mass. During the night, sometimes before midnight, dissolution of the blood islands commenced, and was usually in full progress by next morning. In a fluid medium, or in plasma cultures in which the usual liquefaction of the plasma had occurred in the neighbourhood of the explants, the greater part of the centres of the cultures disappeared, and floating about in the fluid were innumerable small round cells. I shall refer to these floating cells, because of their great number, as "clouds." Examination of dense parts of a cloud at this stage gave the impression that hæmoglobin was present, but the slight yellowish tinge was not sufficiently definite for certainty. The cells still attached to the cover glass consisted of epithelium, mesenchyme, some wandering cells, and a few primitive blood cells. By the following morning the cells in the cloud began to show a decided tendency to elongate, approaching the definitive form of red blood corpuscles, and hæmoglobin was evident. The attached part of the cultures now showed an increase in the number of wandering cells. During the next day or two the erythrocyte-like form of the cloud cells became accentuated, the presence of hæmoglobin frequently became obvious to the naked eye, the culture sometimes looking like a drop of rather dilute blood, and the number of wandering cells increased enormously.

Histological Development of the Cultures.

The primitive streak, at explantation, consists of a mass of young mesenchyme covered by endodermal epithelium below, and by the epiblast above. It is uncertain whether this epiblast, particularly in the posterior part of the streak, should be regarded as presumptive ectoderm or as mesoderm, destined

in the latter case to migrate down into the blastocoele and join the mesenchyme being formed from the primitive streak.

(1) *From Explantation to the Beginning of Dissolution of the Blood Islands.*—The behaviour of the tissue immediately after explantation varied, but there was always a certain amount of contraction and frequently the fragment became a small rounded nodule. There followed a latent period which might last as long as twenty-four hours, at the end of which cells began to emigrate. Sometimes the culture spread as a whole, producing a thin sheet, not very much thicker in the middle than near the periphery. In other cases the main mass of the culture continued as a solid or vesicular nodule, from which isolated mesenchyme cells and wandering cells emigrated, often with sheets of epithelium. Frequently the outwandering was purely epithelial, mesenchyme and wandering cells not appearing till later. Among primitive streak cultures, spreading of the fragment as a whole seemed to occur more readily, the more posterior the position in the embryo from which the explant was taken.

The following account refers to cultures of posterior halves of primitive streaks in which the explant had spread as a whole, because it is only in such cultures that anything could be seen of the changes proceeding in the important central parts of the culture.

When outwandering was well started, the centre of the culture consisted of a dense mass in which the outlines of individual cells could not be seen in life. The dense mass was an hæmangioblast; its structure was studied in stained sections and in whole mounts of cultures of cut up posterior halves of primitive streaks, in which the tiny explants were sufficiently small and thin to allow examination of their central regions. Whole mounts of such cultures, figs. 1 and 2, Plate 22, showed that the hæmangioblasts were surrounded by a flat sheet of large and often very strongly vacuolated epithelial cells, fig. 1; this sheet had the appearance of a syncytium in which no cell boundaries could be detected. The hæmangioblast cells were clumped together in a dense mass. In stained preparations they showed clear cell boundaries; these were not visible in life, and had doubtless been rendered apparent by the shrinkage caused in fixation. Even in the stained material areas were present in which cell boundaries were not to be seen, fig. 1, Plate 22. The cells were much larger than the primitive blood cells to which they gave rise. The cytoplasm was basophilic, but not as intensely so as the primitive blood cells, and areas of many of the cells were faintly eosinophilic. The basophilia appeared to be greater than that of the epithelial cells, but this may have been merely an expression of the greater thickness of the hæmangioblast cells. Many of the

cells had the form of irregular polygons, but others tended to be flattened; this applied especially to those near or on the edge of the mass, while cells within the mass often tended to become flattened between other, more polygonal cells. It was a definite characteristic of the cells that they always tended to flatten against a surface, whether the surface were the edge of the hæmangioblast, of the surrounding cells, or of the cover glass. This tendency is of interest for two reasons: (1) it appears to be an important factor in the differentiation of the first vascular endothelium; (2) its disappearance, and its replacement by a different quality of the cells, is one of the principal changes involved in the dissolution of the blood islands.

The nuclei of the cells were large, clear and contained one or two rather large nucleoli of irregular form. That cell multiplication proceeded actively was shown by the presence of many mitoses, fig. 2. The larger chromosomes had the long slender form usual in the fowl.

The majority of hæmangioblasts showed areas of cellular degeneration, and in these necrotic cells the nuclei became structureless, and intensely basophil, while the cytoplasm disintegrated. The areas of degeneration are usually small, and seemed not to affect the remainder of the hæmangioblast; obviously healthy hæmangioblasts, with many cells in mitosis, often also contained degenerate regions, fig. 2.

Sections, fig. 4, Plate 23, of hæmangioblasts developing *in vitro* confirmed the observations made on whole mounts. In addition, they showed more clearly that the hæmangioblasts may be many layers thick, and that the cells tended to be flattened against the cover glass. A point of some importance, which was not to be discovered from whole mounts but which sections revealed, was the presence of a thin layer of cells covering the hæmangioblast on both sides. Since endothelium developed in many cultures, these investing layers may in some cases represent early stages in its differentiation. Whether endothelium was present or not, however, there was usually a mantle of epithelium investing the hæmangioblast on both sides, and distinguishable from endothelium by its continuity with the emigrated epithelium surrounding the hæmangioblast, by its thickness, and by its often having several layers.

(2) *The Development of Endothelium.*—With the development of endothelium, the hæmangioblast stage comes to an end and that of the blood island begins.

It is not possible to say whether every hæmangioblast developing *in vitro* formed endothelium, but at least a great many did so. Whole mounts of a number of cultures provided evidence of the mode of development of the endothelium. It has been said that the cells of the hæmangioblast tended to

flatten upon surfaces, whether the surface were the cover glass, the hæmangioblast itself, or the surface of other cells in the mass. The development of endothelium is illustrated in figs. 6 and 7, Plate 24. The tendency of the peripheral cells to flatten, fig. 6, became progressively more marked, fig. 7, as the central cells became smaller and more intensely basophil.

Frequently, particularly in plasma and extract cultures from the posterior half of the *area opaca* or of the *area pellucida* lateral to the posterior half of the primitive streak, there appeared quite definite capillary vessels containing blood cells, fig. 8, Plate 24, which usually, if not always, developed hæmoglobin, though they often became very abnormal (see below). Endothelium was not always formed as a continuous sheath enclosing cells; it might form an incomplete fenestrate membrane, or it might be in the form of narrow ribbons winding quite irregularly inside the mass of blood cells of an incompletely dissolved blood island, fig. 3, Plate 23.

It is evident from the fact that the primitive blood cells were usually liberated into the medium that the endothelial and epithelial sheaths in which they were enclosed became ruptured when the dissolution of the blood islands occurred.

(3) *The Dissolution of the Blood Islands.*—During the second night after explantation the area covered by the outwandered cells increased and, if they were not already numerous, mesenchyme and wandering cells became prominent. The dissolution of the blood islands usually began during the same night, but might be earlier or later. The cells composing the islands became smaller, and showed a significant change in shape. The tendency to flatten upon surfaces decreased after the differentiation of the endothelium and finally disappeared, while the cells began to acquire a spherical form. Sections suggest that they tended to retain contact with one another for a time by means of processes, but this was a temporary phase. Soon the more superficial cells of the mass became completely rounded. In this stage the blood island, examined alive, looked like a pile of round, glistening grapes; often, if the culture were given a slight jar, some of the rounded cells were seen to fall off the mass and to sink down in the medium, which was generally liquefied. In the centre of the blood island its thickness and the rounding of the cells made it impossible to study details of structure; it was necessary either to work with sections, fig. 5, Plate 23, or, in whole mounts, to examine the thin peripheral region in which blood island cells which were attached to the glass were not obscured by masses of other cells below them. Near the edges of the blood islands such cells often formed a pseudo-epithelium, fig. 3, Plate 23; that it was not a true epithelium was shown by the fact that the cells

were separate from one another, not forming a continuous sheet. In sections, fig. 5, Plate 23, through the centre of a dissolving blood island, it could be seen that the cells were now no longer flattened upon one another but were rounded, that cell outlines were clear, and that there were often clefts between the cells. While the process of rounding up was going on the cells seemed to become more intensely basophil, but in spite of this there were areas in their cytoplasm which were less basophilic than others, and which even stained faintly with cosin. The nucleus had become somewhat larger compared with the cytoplasm, but its other characters had not greatly changed.

The free, spherical cells, falling away from the attached part of the cultures formed "clouds." They were now primitive blood cells.

Histogenesis of Blood and Wandering Cells.

The primitive blood cells were small and probably amœboid ; at least, after fixation they frequently showed pseudopods and in life amœboid cells of similar appearance, but of uncertain origin, were frequently seen (see below, under "Wandering cells"). The primitive blood cells had a rounded nucleus enclosed in a quite small amount of basophil cytoplasm. The nucleus contained one or two nucleoli of somewhat irregular form, and usually several tiny granules of chromatin. In spite of its general basophilia, the cytoplasm often contained small weakly eosinophil areas resembling those already noted in earlier stages. Some cells may now show the eosinophil structure which Dantschakoff (1908) calls the "Hof," a term for which the nearest English equivalent, in the sense in which the German word is used, is perhaps "focus," as in the phrase "focus of disease." It is described by Dantschakoff as a more or less spherical region of eosinophil cytoplasm which, increasing in size, indents the nucleus, making it kidney-shaped. I confirm Dantschakoff's description in both cultures and normal embryos, save that I find the focus to be less regular in form than is indicated by her figures, and that there may be one large and several smaller foci in a single cell.

Camera lucida drawings showed that the primitive blood cells tended to be somewhat smaller than the corresponding cells *in vivo*. They proliferated rapidly, and the chromosomes were still long and thin, as in the blood island stage ; a resting cell is shown in fig. 9, Plate 25.

A.—Erythrogenesis.

The development of erythroblasts took place almost entirely among cells floating in the liquefied medium. The histogenesis was studied mainly from

smears, but the results so obtained were as far as possible checked by reference to the Zenker formol fixed whole mounts and sections. In most cultures, although nearly all the cells derived from the blood islands sank into the fluid, some remained attached to the tissue on the cover glass, or enclosed in capillary vessels from which they were unable to escape. These "relict" cells were not a reliable foundation for the study of histogenesis because, when developing in enclosed spaces in the culture, they tended to become abnormal, and often remained undifferentiated or in the first stage of erythrogenesis, long after the cells floating in the fluid had become advanced erythroblasts. They could, however, be used for certain purposes as a check upon the cells in the smears.

The histogenesis of the red cells may be considered in three stages. As the living cells were very translucent and gave little information, the following account is based upon fixed and stained preparations.

Stage (1).—This stage, fig. 10, Plate 25, was characterised by increase in size of the small primitive blood cells. The cells retained their rounded form, and did not show pseudopods, while the cytoplasm remained basophil but was not completely homogeneous. It was usually uneven in texture, and contained an eosinophilic focus or foci, as described in the primitive blood cells, but now becoming more prominent. These foci were sometimes irregular in distribution and form, sometimes streaky and lying along curves concentric with the surface of the cell, and sometimes more or less spherical. The nucleus, probably owing to the relative and absolute increase in the quantity of cytoplasm, was now no longer indented, but was spherical. The cytoplasm of the relict cells in the attached cultures was usually more homogeneous, except when the cells were obviously unhealthy. It must be remembered, however, that it was more difficult, on account of the plasma present, to obtain a satisfactory stain of the attached culture than of the cells in the smears. The eosinophilic focus was not seen in all cells, but it was found in a large number.

The appearance of the nucleus at this stage depends to some extent on the fixation. It was always round or oval, but in the smears (fixed by methyl alcohol) it showed a brilliant red-purple colour and was full of red-purple granules of irregular form and indefinite contour, which seemed to be united to one another by threads of the same material, forming a loose and irregular network. Between these large granules were very numerous tiny pinkish granules, with the result that the whole nucleus had a smudgy appearance. Corresponding cells in the attached parts of the cultures (Zenker formol) showed a very different picture. The reddish granulation was not to be seen,

and the nuclei were vesicular with irregularly scattered basophil chromatin granules, and, in at least some, and probably in all cells, there were one or two rather faintly staining basophil nucleoli. There can be no doubt that the nuclear appearance seen in the smears was an artefact, and that the picture shown by Zenker fixed material resembled more closely the condition in life. During this stage cells in mitosis were frequently found, and it is evident that differentiation may proceed through several generations; whether the entire differentiation process was ever completed in a single cell generation is uncertain. The chromosomes which were previously long and thin became at this stage short and thick, and lay so close together that boundaries between adjacent chromosomes could not be distinguished. This condition was seen in all mitoses until cell division ceased.

Stage (2), fig. 11, Plate 25.—During the second stage the form of the cell altered, and changes occurred in the nucleus. Elongating along one axis the cell gradually became equi-oval in form, with blunt ends, and at the same time, though generally after the attainment of a broad oval form, the basophilic substance decreased in quantity, and began to be replaced by the eosinophilic substance which is generally, and probably correctly, regarded as being hæmoglobin. It must nevertheless be noted that hæmoglobin was present at earlier stages than this. As this change proceeded the cells at first acquired (after Giemsa) a curious grayish-pink-purple colour, and later became pale pink. The texture of the nucleus appeared at first glance to be homogeneous, but careful examination revealed a faint and loose reticulum of cytoplasmic strands, or sometimes there might be several small vacuoles embedded in a more or less homogeneous pink ground substance. The nucleus became relatively smaller, but in many cells remained for a time large enough to touch both sides of the elongated cell body. The chromatin took the form of a coarse and, at first, irregular network, which became more regular in arrangement and tended to concentrate at the periphery of the nucleus, where it might resemble the spokes of a hub-less wheel. Nucleoli were still present in some cells at least. The reddish substance, so prominent in earlier stages, became less so, and now no longer obscured the chromatin, even in smears. If mitoses occurred at all after the cells had begun to elongate, they were rare; hence it is probable that the differentiation processes of the second and third stages occurred within the lifetime of single cells, without the occurrence of cell divisions.

There was considerable variation in the time relations of the process. The cells might lose the basophil substance before the eosinophil substance

appeared, so that the cytoplasm became very pale and had the appearance of a loose reticulum traversing a space filled with a fluid or some other homogeneous substance. In many cultures there were round cells which had not yet begun to elongate, with very pale or slightly eosinophil cytoplasm and small nuclei of the kind described. Whether such cells retained their circular form and never became oval, or whether they became so later, is uncertain.

Stage (3), fig. 12, Plate 25.—I have only once obtained cells which could be called mature erythrocytes; these were in a culture taken from the *area opaca* at a time when hæmangioblasts probably existed. The infrequency was probably due to the fact that the cultures have usually been fixed too soon, because, if kept alive longer, the cells became abnormal. In shape the differentiated erythrocyte was equi-oval, and strongly eosinophil. The nucleus was roughly oval or somewhat irregular in form, quite small and shrivelled, darkly basophil, and contained several large chromatin granules. The cytoplasm appeared to be perfectly homogeneous.

Comparison of erythropoiesis *in vitro*, as here described, with the corresponding process *in vivo*, described by Dantschakoff (1908), shows general agreement, and minor points of difference are probably due in part to differences in staining.

The Development of Hæmoglobin.—In older cultures, containing advanced erythroblasts, the presence of hæmoglobin is frequently obvious, and can often be detected with the naked eye. Professor Keilin examined a number of the cultures spectroscopically, and found the characteristic spectrum of hæmoglobin. The earliest culture in which it was recognised consisted of a dense mass of cells just released, or just about to be released, by the dissolution of a large blood island. The cells appeared to be in all respects normal young primitive blood cells; there was nothing suggesting that the dissolution of the blood island had been abnormally delayed.

No trace of hæmatoporphyrin, or of any other hæmoglobin derivative, could be detected.

Abnormal Forms among the Erythroblasts.—Figs. 13, 14 and 15, Plate 25.—Shipley (1915–16), describing hæmatopoiesis in his cultures of the *area opaca*, states that all but a few of the erythrocytes formed were more or less abnormal. The same applies to the red blood cells formed during the course of the present experiments, but the degree of abnormality was in most cells not high, careful examination being required to reveal that a particular cell was in some respect not quite normal. Most cultures, on the other hand, contained some cells which were very abnormal indeed, and in some cultures this was true of all or nearly all.

The commonest abnormality of form, as distinct from purely necrotic changes, consisted in the development of vacuoles within the cytoplasm. A large percentage of late erythroblasts, when examined closely, could be seen to contain one or a few small vacuoles, and this comparatively trifling deviation from the normal was connected by all transitions with cases in which the cell was reduced to a mere thin-walled vesicle, fig. 13, Plate 25, or complex of vesicles enclosed by the outer wall of the cell, fig. 14, Plate 25. The nucleus of cells in this condition was strongly compressed either against the cell wall or in a dissepiment between vacuoles. The material in the vacuoles did not stain with Giemsa and so was not hæmoglobin, and it had not the appearance of fat. It is interesting that a moderate vacuolation neither prevented the development of hæmoglobin nor seriously impeded the development of the typical erythrocytic form. Extreme vacuolation caused the cell to remain spherical; its effect on the development of hæmoglobin is uncertain. Cells reduced to a bag by a single large vacuole may occasionally be seen in normal embryos.

Another abnormality, often associated in the same cell with a limited vacuolation, was the formation of erythroblasts which were drawn out at one or both ends into long pointed processes which tended to show beading. Both these cells and the vacuolated cells often showed, in addition, curvature of the whole cell into a crescent form, fig. 15, Plate 25.

Otherwise, normal erythroblasts with two nuclei were not uncommon, and in highly vacuolated cells there might be three or even four.

The various abnormalities, and especially vacuolation, show a definite association with unfavourable conditions in the culture. Frequently, as has been said, masses of primitive blood cells were unable to become free from the attached part of the culture because they were enclosed in capillaries or between sheets of cells. In these conditions it very frequently happened that all the cells in the mass became intensely vacuolated, while those which had been able to escape into the fluid continued normal development.

B.—Wandering Cells (Histiocytes, Polyblasts).

The appearance of wandering cells at an early stage in the development of the cultures has been mentioned. After the dissolution of the blood islands, when there was a floating cloud of free cells, many of these were found to be wandering cells. Present in only small numbers at first, the wandering cells multiplied enormously in later stages, and sometimes even outnumbered the

erythroblasts. They were present throughout the attached part of the culture, as well as in the floating cloud. In later stages they became very actively phagocytic, and had the structure shown in fig. 17, Plate 25. Seen in life, they were cells filled with glistening droplets and varying considerably in size, included among them being the largest free cells present. Studied after fixation and staining, they showed the following characters: the cytoplasm was much vacuolated, being filled with many small vacuoles, and many cells were engorged with the remains of phagocytosed material, chiefly erythroblasts and primitive blood cells. In many cells, particularly in those which had not been actively phagocytic, the nucleus was a fairly regular, small, oval, eccentrically placed structure, containing one or two large nucleoli and particles of chromatin. In other cells, and especially in the larger, more engorged, and probably older cells the nuclei had the most varied and fantastic shapes, lying compressed between the vacuoles with which the cell body was distended. In such cases it was difficult to make out anything of the nuclear structure, but it seemed to resemble that of the more regular nuclei so far as its peculiar form and its compression allowed. The nucleus of the cell shown in fig. 17 is of regular form compared with that seen in many cells.

The origin of the wandering cells remains uncertain. Examination of living cultures frequently showed all transitions between large engorged and vacuolated phagocytes, and small amoeboid cells with few or no vacuoles or droplets. Some of these small cells so closely resembled primitive blood cells as to suggest that the wandering cells were derivatives of the blood islands; but whether these were one source of wandering cells or not, they were certainly not the only source, for exactly similar wandering cells appeared in large numbers in cultures in which there was no hæmatopoiesis. Further, in such cultures one sometimes finds, associated with the wandering cells, just the same small, round, clear cells as seem to be the young stages of the wandering cells in cultures containing blood islands and primitive blood cells. It is therefore probable that wandering cells are not derived from the blood islands but from the small round cells which closely resemble primitive blood cells and which originate from the mesenchyme in some manner which remains unknown.

C.—*Endodermal Wandering Cells.*

Suspecting that certain cells in the cultures were identical with the endodermal wandering cells, of epithelial origin, described by Dantschakoff (1908) and others, I made a series of cultures of the endodermal epithelium. These

cultures usually contained a certain amount of mesoderm, for it was difficult to separate it completely from the endoderm; the suspicion was, however, confirmed by finding the suspected cells present in great numbers in cultures of endoderm, while the mesodermal wandering cells were much less numerous or altogether absent. The endodermal wandering cells had a central mass of glistening droplets, presumably of yolk, and a lobular pseudopodium of very clear cytoplasm, there being usually a sharp line of demarcation between the clear cytoplasm of the pseudopod and the heavily yolk-laden endoplasm. They degenerated within two or three days, as might be expected from their normal history *in vivo*.

D.—“*Large Lymphocytes.*” (Fig. 16, Plate 25.)

Cells identical with those which Dantschakoff (1908) calls large lymphocytes were undoubtedly present in the smears. While not convinced of the identity of these cells with the large lymphocytes of later stages, the name is retained provisionally.

The number of these cells varied greatly from culture to culture; they were always far fewer than the erythroblasts, and sometimes seemed to be absent altogether. They were round cells, about the same size as the larger round erythroblasts, but differed from the latter in having a relatively very large nucleus surrounded by a narrow rim of cytoplasm. The cytoplasm was basophil, but might contain one or more areas resembling the eosinophilic focus of erythroblasts; this is clearly shown, for the normal embryo, in Dantschakoff's figures. The nucleus was round and, in most cells stained in smears, its structure was obscured by a mass of light red granules joined together as a net. There is little doubt that this is an artefact like the similar substance found in smear-stained early erythroblasts; it always seemed to be present, but often was only very lightly stained so that the nuclear structure could be studied. The nucleus was then seen to contain one or two rather small nucleoli of irregular form, and often a few small fragments of chromatin, the nucleus as a whole having a curiously empty appearance. I have made no detailed study of the histogenesis of these cells, but there is no doubt that they, like the erythroblasts, are derivatives of the blood islands; they might be described as an enlarged form of the primitive cell.

E.—*Other Leucocytes.*

Dantschakoff (1908) described cells with eosinophilic granules as appearing in the extra-vascular spaces of the yolk sac trabeculae at about four to five days.

of incubation. Cells which are almost certainly young stages in the development of these eosinophils are present in one culture and possibly in three others.

In the same paper Dantschakoff described dwarf lymphocytes and thrombocytes as differentiating from primitive blood cells. Whether these are present in the cultures or not remains uncertain.

PART 2.

The Distribution of the Hæmatopoietic Cells.

The stage of development of the embryos from which I have, in the great majority of experiments, obtained material for cultivation, was that at which there is a fully-developed primitive streak, with or without a head process, in a pear-shaped *area pellucida* with no head fold. I have also obtained development of red corpuscles from stages well before the appearance of the primitive streak, but in these early stages I have not yet made any attempt to delimit the areas of the blastoderm containing the hæmatopoietic cells.

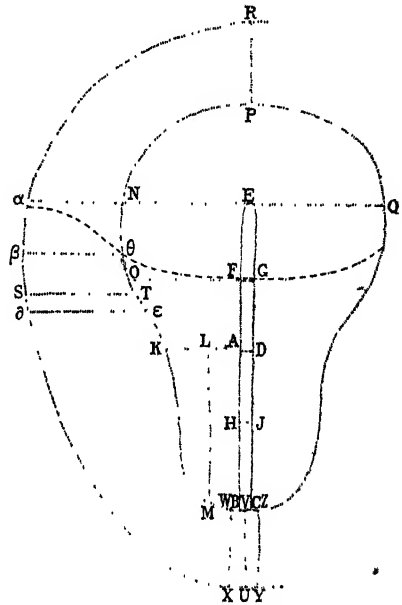
In the following paragraphs the letters in brackets refer to the areas indicated in fig. 18.

At the primitive streak stage at which most of the experiments have been made, a very large number of experiments have shown that cultures of the posterior half of the primitive streak (ABCD) almost invariably produce great numbers of red blood cells.

To find the distribution of the hæmatopoietic cells along the length of the primitive streak the following experiment was carried out. Cultures were made of the anterior quarters (EFG) of the primitive streaks of twenty-two embryos; three of these developed a little blood, two were doubtful, and the remaining seventeen developed no blood at all. Eleven cultures were made of the second quarters of the primitive streaks from the same embryos as the last group (FADG); all showed vigorous development of blood. A fragment was cultivated from the posterior end of each of twelve primitive streaks, the fragments varying in size from eighths to quarters (HBCJ); all produced blood. It may therefore be concluded that hæmatopoietic cells are present in the whole of the posterior three-quarters of the primitive streak, but that they are absent from the anterior quarter except, perhaps, in its posterior end.

Eighteen cultures were made of that part of the *area pellucida* which lies lateral to the posterior half of the primitive streak, between it and the *area*

opaca (KMBA). Blood developed in twelve of these cultures, probably in three others, was absent in one and probably in another, and one was quite doubtful. Nearly all the cultures showed appearances known to me as early stages in hæmatopoiesis, but are counted as doubtful unless cells containing hæmoglobin, or showing the oval form of the late erythroblast, were clearly recognisable. In embryos at this stage it can frequently be seen that the posterior part of the *area pellucida* is divisible into a more opaque, median zone, near the posterior half of the primitive streak, and a lighter more peripheral zone. Twelve cultures were made, six of which consisted of the inner, opaque zone (LMBA), and six of the outer clear zone (KML). Of the former, four showed definite development of blood and two were doubtful, but all showed some signs of hæmatopoiesis. Of the second group, three showed blood, two were doubtful and one was negative, showing no indications of hæmatopoiesis. To find the distribution of the hæmatopoietic cells in the more anterior part of the *area pellucida*, I made thirteen cultures of the *area pellucida* opposite the anterior quarter of the primitive streak (NOFE); blood appeared in four of these, but was absent from the other nine, while in five cultures made from the *area pellucida* opposite the head process of embryos in early head process stages, no blood was developed. Six cultures were made of the *area pellucida* anterior to the primitive streak or head process—that is, from the region of the pro-amnion (PNQ), but none of them developed blood. It may be concluded that hæmatopoietic cells are present opposite the posterior three-quarters of the primitive streak, and may extend a little further anteriorly. The pro-amnion, naturally, does not contain hæmatopoietic cells.



Description of FIG. 18.—Diagram of an embryo in the stage used for the majority of experiments. The broken line indicates approximately the anterior border of the region occupied by hæmatopoietic cells. The peripheral dotted line on left indicates roughly the peripheral limit of the region used for cultures, not the actual limits of the blastoderm, which extends beyond this line. The lettering is explained in the text.

A series of cultures was made from the inner part of the *area opaca*, where the greater part of the *area vasculosa* later appears. This region was divided into four, as shown in the diagram, and the four quadrants were cultivated separately. Thirteen cultures of anterior quadrants (RSTP) all gave negative results except one, in which blood developed, the remaining twelve being free from blood. Thirteen cultures of posterior quadrants (SUVT) gave development of blood in ten cases, while one was doubtful and two negative. Fourteen cultures were made of strips of *area opaca* from immediately behind the primitive streak (WXYZ); blood appeared in all. In order to demarcate more accurately the region occupied by the blood-forming cells, twelve cultures were made of rather narrow strips of *area opaca* taken from a position such that their anterior ends were opposite the widest part of the *area pellucida* ($\alpha\beta\theta\kappa$); of these six produced blood and six did not. Hence, it appears that the region of the *area opaca* opposite the widest part of the *area pellucida*, and approximately opposite the anterior end of the primitive streak, contains the boundary between the region occupied by hæmatopoietic cells, and that into which they have not yet penetrated. To confirm this, four cultures were made of strips of *area opaca* from a position ($\beta\delta\epsilon\theta$) immediately behind that represented in the last series; all formed blood.

It may be concluded from these experiments that the hæmatopoietic cells are distributed over the whole of that part of the embryo which lies behind the broken line in fig. 18.

DISCUSSION.

(1) *The Determination Problem.*

A.—*The Hæmangioblasts.*—The first stage in hæmatopoiesis is the formation of the hæmangioblasts. Their formation is a differentiation process depending upon the activity of their component cells, and the question arises whether it is a self-differentiation.

It is evident from the cultures herein described that the differentiation of the hæmangioblast is not dependent upon the specific activity of any organ of the embryo. This conclusion follows equally from the explantation experiments of Slonimski (1930 *a*, 1931). *In vivo*, hæmangioblast formation might be enforced upon the mesenchyme by some condition existing either in the primitive streak or in the *area vasculosa*. The first would mean that mesenchyme cells at the place, and at latest very shortly after the time, of their origin, become specifically determined for hæmangioblast formation, or at least strongly biassed in

that direction. In this case the cells which form the hæmangioblasts would do so under the influence of factors intrinsic in themselves; in other words, hæmangioblast formation would be a self-differentiation. If the hypothetical condition acted in the *area vasculosa* it would either not exist in the primitive streak cultures, or else the latter must establish an internal environment closely resembling that in the normal *area vasculosa*. The internal environment could hardly be produced by a particular structure of the *area vasculosa*, because the only resemblance between its structure and that of the cultures is the presence of the hæmangioblast. If it were a particular physiological condition, it must be one due to the activity of a particular kind of cell. This hypothesis must be rejected for two reasons. Firstly, it requires the assumption, without evidence, of specificity in an unidentified cell to avoid making the same assumption in the case of the presumptive hæmangioblast cells. Secondly, the mesenchyme is the only tissue which belongs in common to both primitive streak and *area vasculosa*, because it migrates from one to the other. There is no evidence for any similar migration of ectoderm or endoderm. Hence, to save the hypothesis it would have to be assumed that the internal environment, normally created by an unidentified cell in the *area vasculosa*, was in the cultures created by a different unidentified cell which normally displays no such activity.

It may therefore be concluded that the mesenchyme cells of the primitive streak are caused to form hæmangioblasts, not by conditions extrinsic to them, but by the action of factors intrinsic in them. In other words, *the mesenchyme cells which form the hæmangioblasts must be regarded, even while they are still in the primitive streak, as self-differentiating cells, predetermined, or at least strongly biassed, towards the formation of the group of cell types which are derived from the hæmangioblasts.*

This involves further implications. According to the view which is generally held at present, the hæmatopoietic process begins in a previously undifferentiated mesenchyme, all of whose cells are equivalent to one another. This view is evidently inconsistent with the conclusion just stated, and must therefore be abandoned, at least for primary hæmatopoiesis in the chick embryo. The work of Slonimski (1930 *a*, 1931), to which reference has been made, indicates that a similar conclusion must apply to the corresponding process in Amphibia. Further support is found in the works of Federici (1926), Goss (1928), Slonimski (1930 *b*) and Stohr (1931). These authors, using young embryos of various Amphibia, performed experiments which consisted essentially in removing the hæmatopoietic region, and by this means a number of

larvæ were obtained which, although without erythrocytes, in some cases lived almost to metamorphosis, but failed completely to produce red blood cells, making no attempt to compensate for the loss by any regenerative process.

It is important at this stage to mention two points. Firstly, the term "biased" is used here in order to avoid the term "determination," the use of which suggests irreversible predestination. The German school of experimental embryologists has shown that cells which can, when isolated, self-differentiate in the expected manner may nevertheless be caused to differentiate in quite other ways if placed in the appropriate conditions. It is possible that the presumptive hæmangioblast cells of the primitive streak might similarly alter the line of their differentiation if they were subjected to suitable treatment. Secondly, the fact that some mesenchyme cells do not take part in hæmatopoiesis does not mean that they, or their descendants, may not do so at later stages or under different conditions.

B.—*The Endothelium*.—It is theoretically possible either (1) that the presumptive endothelial cells in the hæmangioblast are constitutionally identical with all the other hæmangioblast cells, and that they suffer endothelial differentiation because of their tendency (shared with all other hæmangioblast cells) to flatten on surfaces, and because of the pressure of the developing intravascular fluid; or (2) that they were specifically biased or even finally determined for endothelium formation prior to the development of the hæmangioblasts, and have taken a superficial position because their innate constitution forces them to do so. While it is impossible to decide between these alternatives, the first accords with the histological picture and involves fewer and less improbable assumptions. But whichever is correct, it is important to remember that the endothelium retains, at least for a time, the power of giving rise to new erythroblasts (Sabin, 1920; Dantschakoff, 1908, 1909).

C.—*The Erythroblasts*.—The time of first appearance of hæmoglobin sets a later limit to the time of determination of erythroblasts. Hæmoglobin is undoubtedly present in the elongating erythroblast, but there is no doubt that its first appearance is considerably earlier than this. Sabin (1920) found that hæmoglobin could be seen as a yellow colour in the living cells of undissolved blood islands, but not in the earlier hæmangioblast ("angioblast" stage. Slonimski (1927 *b*), using a micro-chemical benzidin method (1927 *a*) identified hæmoglobin in the peripheral zone of the *area vasculosa* at six to seven somites. In a culture consisting of a large blood island just at the stage of

dissolution, Professor Keilin, as stated earlier in this paper, found hæmoglobin definitely present. From these isolated facts it appears that hæmoglobin originates during the time which elapses between the development of the endothelial walls and the dissolution of the blood islands. The final determination of the presumptive erythroblasts must therefore occur during or before the blood island stage. No early limit can be definitely fixed, but if the peripheral cells of the hæmangioblast, which form endothelium, are constitutionally identical with all other hæmangioblast cells, the presumptive erythrocytes must be determined towards erythrocytogenesis after or during the development of endothelium, since, by hypothesis, they were previously equally liable, according to their position, to either erythrocytogenesis or to endothelium formation.

It should be noted that the erythrocytes differentiating in the cultures are doubtless the first or primary erythrocytes (Dantschakoff, 1908), for the definitive erythrocytes, which differ very little from the first series, do not appear *in vivo* until after four or five days' incubation.

(2) *Wandering Cells.*

Since the wandering phagocytic cells appeared in all cultures containing mesoderm, whether hæmatopoiesis occurred or not, it is clear that they are connective tissue cells, histiocytes, rather than blood cells.

They appear first on the day after explantation, which corresponds to the second day of incubation. Now, Dantschakoff (1909), in a very complete study of the development of the connective tissue of the chick embryo, states that the mesenchyme is at first a uniform reticulum of stellate cells, and that wandering cells do not appear until the end of the fourth or the beginning of the fifth days of incubation. It is thus probable that the wandering cells in the cultures are precocious, their presence being not merely the result of the emigration of already existing wandering cells from the explant, but of their new formation as a reaction to the conditions of life *in vitro*.

(3) *The Histogenesis of Endothelium.*

Sabin (1920), in her account of the living *area vasculosa*, states that the plasma of the first vessels is formed in the solid hæmangioblast ("angioblast") by the liquefaction and destruction of the cells, and that the endothelium is formed, not by flattening of the peripheral cells but by the liquefaction of

all but their peripheral regions. From the study of fixed preparations, all other authors have concluded that the endothelial cells are the flattened peripheral hæmangioblast cells ("blood island cells"), and the cultures, as figs. 6 and 7, Plate 24, show, support this view. Nevertheless, special weight should, perhaps, be attached to direct observations of the living organism, and it seems well, therefore, to withhold decision until Sabin's observations shall have been repeated. It is not impossible that both processes may occur.

In conclusion, it is more than a pleasure to express sincere appreciation and thanks for the assistance which I have received from several people during the course of the work, and in particular to Dr. H. B. Fell, who has throughout the work been a never-failing source of help and constructive criticism, especially in the many difficulties of tissue culture methods, in histological technique, and in the preparation of the plates. Thanks are due also to Professor Keilin, of the Molteno Institute, for his spectroscopic examination of the cultures; to Dr. J. S. Niven for valuable help in the preparation of the manuscript, and in other ways; to Professor J. T. Wilson; and to my wife, who worked through many a long day, and actually prepared a number of the cultures upon which this work is based. Thanks are also due to the Royal Society for a grant from the Smithson Fellowship Fund.

Summary.

(1) The differentiation *in vitro* of the blood of the early chick embryo was studied histologically.

(2) The media used were two: plasma and embryo extract, and serum and embryo extract.

(3) Hæmatopoiesis occurred in the great majority of all cultures of any region of the blastoderm behind the level of the anterior quarter of the primitive streak. Hæmatopoiesis may occur in cultures taken from levels slightly anterior to this in the *area pellucida* or in the *area opaca*. Cultures of the entire or fragmented posterior three-quarters of the primitive streak all produce large numbers of advanced, hæmoglobin-containing erythroblasts.

(4) Endothelium develops in many if not in all cultures, but particularly in fragments of the *area opaca* or *area pellucida*. It forms by the flattening of the peripheral cells of the hæmangioblast, but it is not impossible that the excavation process advocated by Sabin may also occur.

(5) The histogenesis of the erythroblasts agrees in essentials with the same process in the normal embryo, as described by Dantschakoff.

(6) Certain abnormalities which commonly occur among erythroblasts differentiated *in vitro* are described. These are chiefly vacuolation, the drawing out of the poles of the cell into long processes, and curvature of the cell as a whole.

(7) In addition to red blood cells and endothelium, cells are found which are identical with those, found in the normal embryo, which Dantschakoff calls "large lymphocytes." In one culture, and perhaps in three others, cells appeared which were probably very young eosinophils.

(8) In all cultures, save those from the pro-amnion, which contained no mesoderm, there appeared epithelium, mesenchyme, and mesodermal wandering cells.

(9) The wandering cells were actively phagocytic, very vacuolated cells with a small eccentric and often distorted nucleus. They originate from mesenchyme, but perhaps also from the primitive blood cells derived from the blood islands.

(10) *Conclusions.*—A.—The primitive streak contains mesenchyme cells which are determined, or at least strongly biassed, towards the development of hæmangioblasts, and therefore of the group of cells, considered as a whole, to which the hæmangioblasts give rise.

B.—The endothelium may be formed by cells already specifically determined for endothelium formation, but it is more probable that it is formed by previously indifferent hæmangioblast cells whose fate is determined by the accident of their superficial position in the hæmangioblasts.

C.—Hæmoglobin is present in the blood island before their dissolution into primitive blood cells. Hence, the presumptive erythrocytes are determined some time before their liberation as primitive blood cells, and probably after the formation of endothelium.

DESCRIPTION OF PLATES.

PLATE 22.

FIG. 1.—A very small hæmangioblast lying on a layer of vacuolated epithelium. Note mitoses, tendency of peripheral cells to flatten, and areas of necrosis. *Explant*: Longitudinal half of rather less than posterior half of primitive streak from an embryo having no head process. The explant was cut into small fragments. Serum and embryo extract, Zenker formol, Giemsa, whole mount.

FIG. 2.—Small hæmangioblast lying on a layer of vacuolated epithelium. *Explant*: Longitudinal half of rather less than posterior half of primitive streak of an embryo having a head process equal to between one-third and one-half of the length of the head process plus primitive streak. The explant was cut into small fragments. Serum and embryo extract, Zenker formol, Giemsa, whole mount.

PLATE 23.

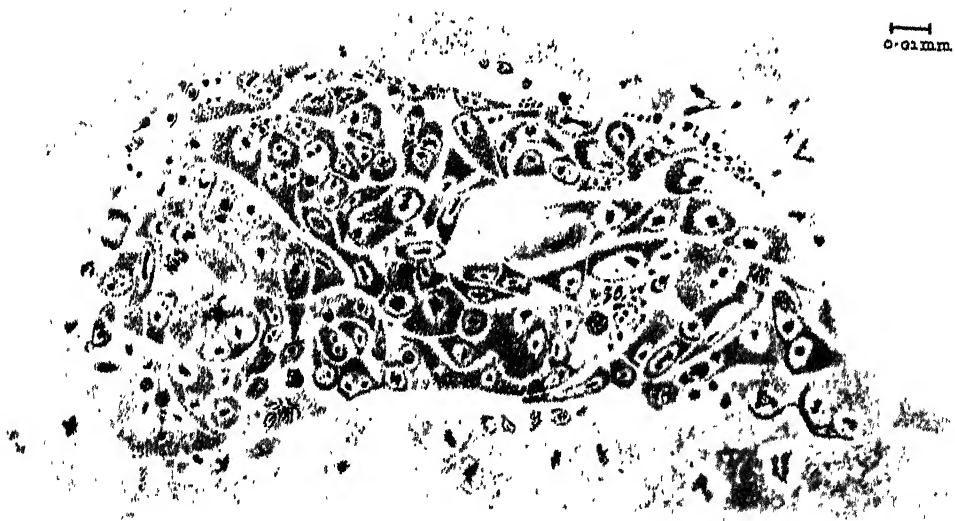
- FIG. 3.—Edge of a culture showing blood island cells attached to the glass, a few primitive blood cells, and endothelium. *Explant*: Longitudinal half of posterior half of primitive streak of an embryo having no head process. Serum and embryo extract. Zenker formol, Domenici, whole mount.
- FIG. 4.—Vertical section of a culture passing through hæmangioblast. Note epithelial layers covering both surfaces, more lightly stained than the hæmangioblast cells. *Explant*: posterior half of primitive streak of an embryo, possibly having a short head process. Plasma and embryo extract, Zenker acetic, Giemsa.
- FIG. 5.—Vertical section of a culture passing through blood islands in dissolution. The cells in the cavity are late blood island cells (at extreme right and left) and primitive blood cells (free in centre). *Explant*: posterior half of primitive streak of an embryo having no head process. Plasma and embryo extract, Zenker acetic Giemsa.

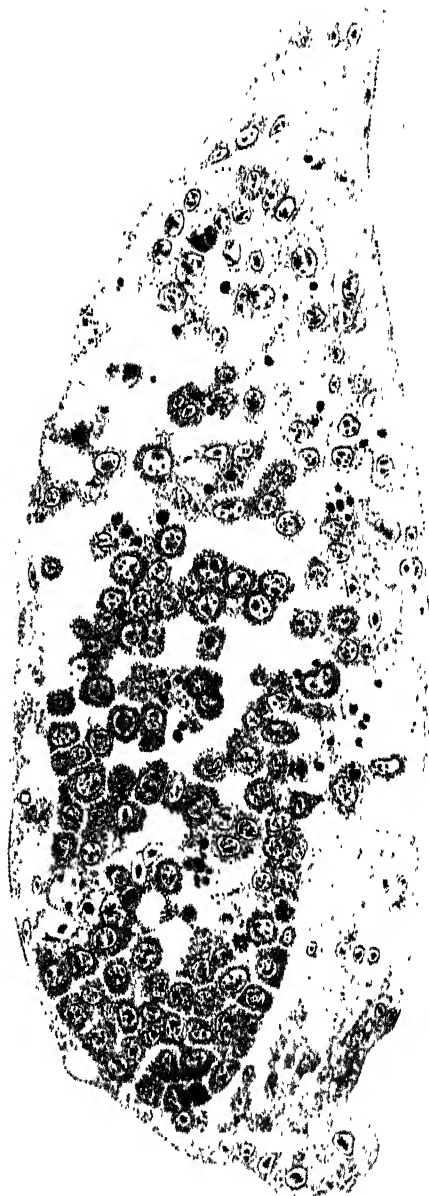
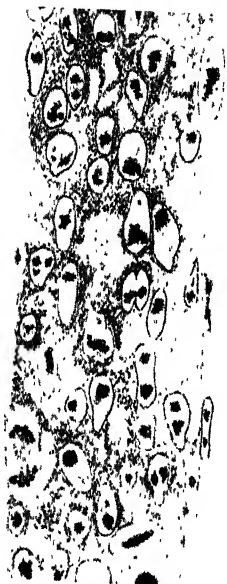
PLATE 24.

- FIG. 6.—Tiny hæmangioblast in a large culture, the remainder of which is not shown. Note flattening of peripheral cells. *Explant*: posterior half of primitive streak of an embryo having no head process. Serum and embryo extract, Zenker formol, Giemsa, whole mount.
- FIG. 7.—Tiny blood island from same preparation as fig. 6, showing more advanced stage in development of endothelium.
- FIG. 8.—Part of a large culture showing capillary vessels containing primitive blood cells or erythroblasts, lying on a vacuolated epithelium. *Explant*: a fragment of the *area opaca* of an embryo having possibly a short head process. Plasma and embryo extract, Zenker formol, Giemsa, whole mount.

PLATE 25.

- FIG. 9.—Primitive blood cell just released from a blood island. The light area in the cytoplasm is the eosinophilic focus ("Hof"). *Explant*: longitudinal half of posterior half of primitive streak of an embryo having a short head process. Serum and embryo extract, Zenker formol, Giemsa, whole mount.
- FIG. 10.—Erythroblast in stage 1. Semidiagrammatic, the cytoplasm was drawn from a cell in a smear fixed in methyl alcohol and the nucleus from a cell in a whole mount fixed in Zenker formol. The light areas are more or less eosinophilic, and the largest is the eosinophilic focus. Plasma and embryo extract, Giemsa.
- FIG. 11.—Advanced erythroblast at end of stage 2. *Explant*: posterior third of primitive streak of an embryo having definitive primitive streak but no head process. Plasma and embryo extract, smear, Methyl alcohol, Giemsa.
- FIG. 12.—Erythrocyte. *Explant*: strip of *area opaca* from embryo having a short head process. Plasma and embryo extract, smear, methyl alcohol, Giemsa.
- FIG. 13.—Abnormal erythroblast containing one large vacuole. *Explant*: posterior halves of two primitive streaks from embryos without head processes. Plasma and embryo extract, Zenker formol, Giemsa, whole mount.
- FIG. 14.—Abnormal erythroblast containing several vacuoles. From the same preparation as fig. 13.
- FIG. 15.—Abnormal erythroblast showing curvature and pointed ends. *Explant*: inner region of *area pellucida* opposite posterior third of primitive streak of embryo having no head process. Plasma and embryo extract, smear, methyl alcohol, Giemsa.





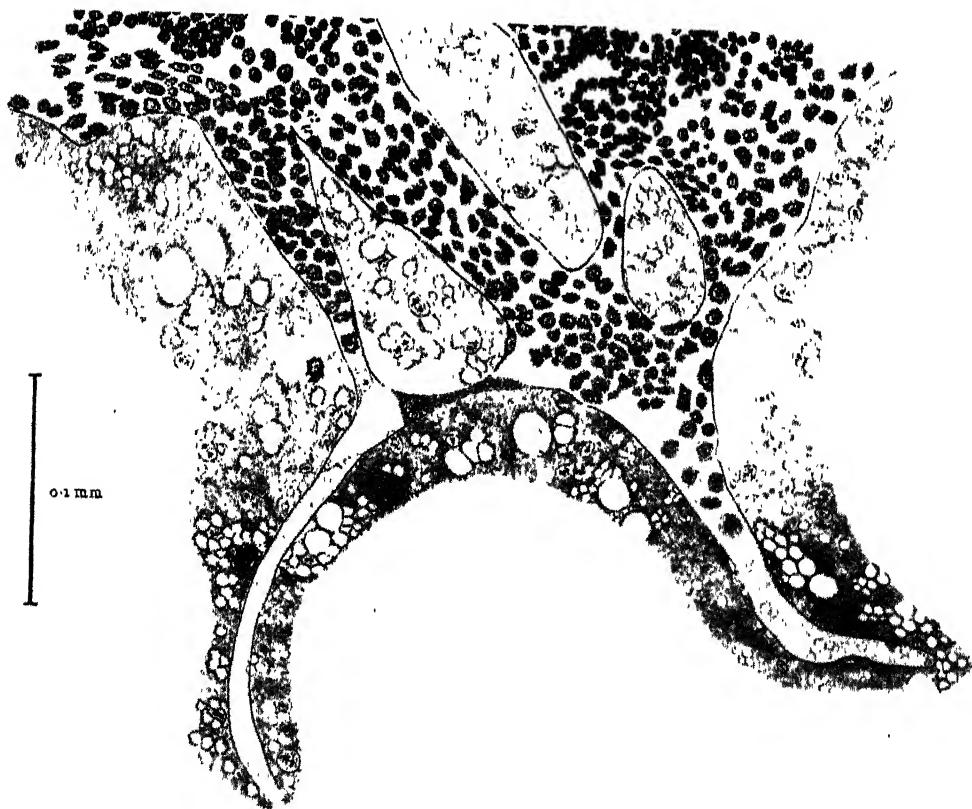


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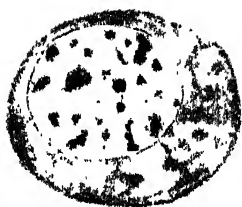


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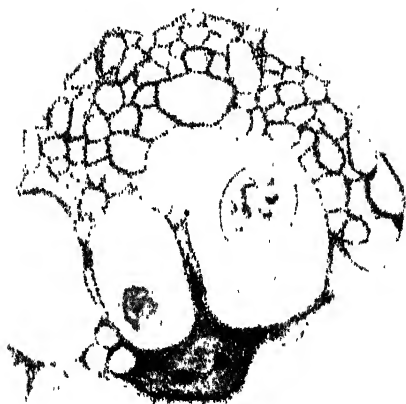
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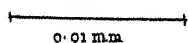
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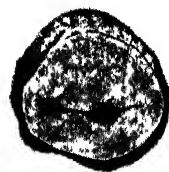
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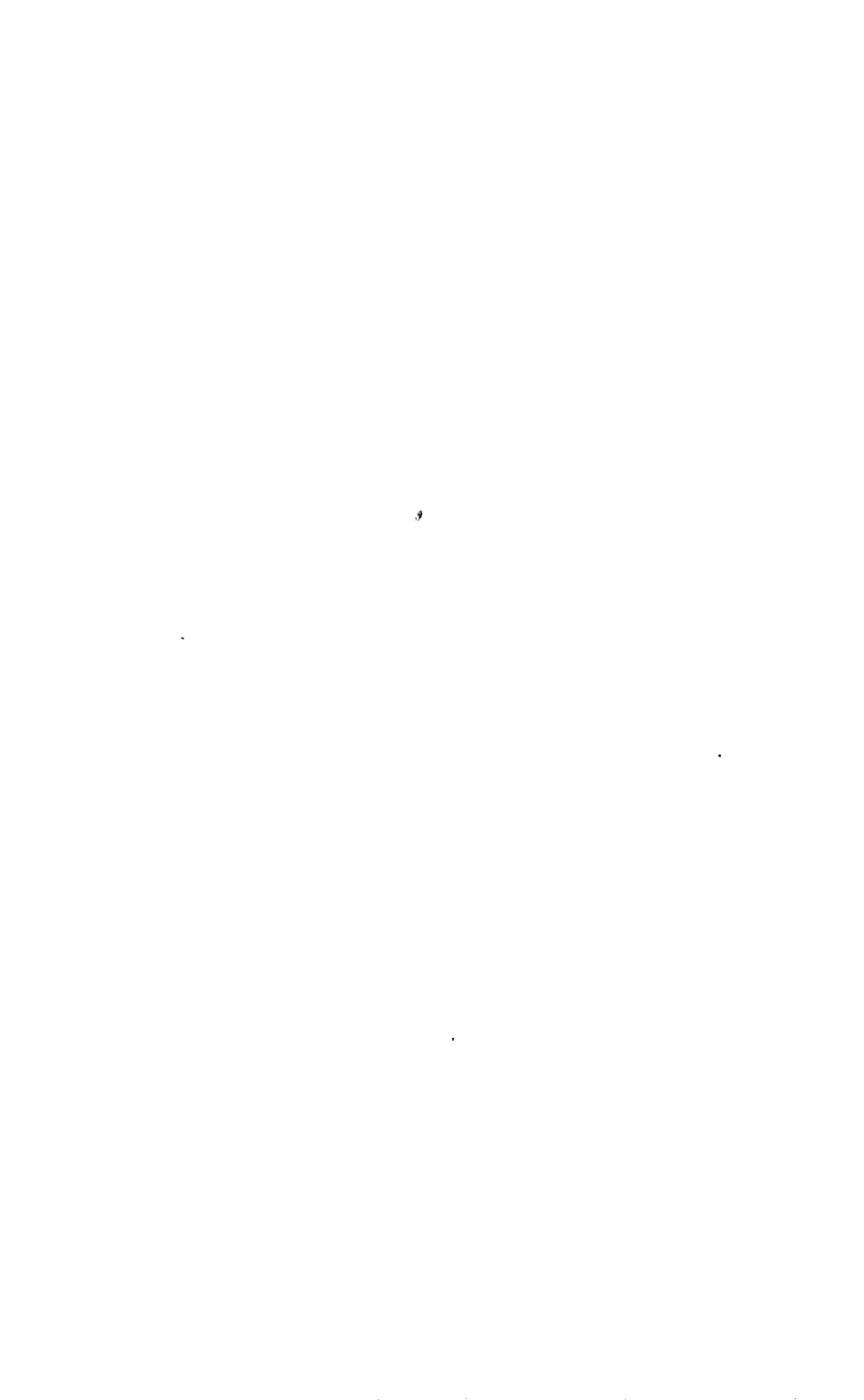


FIG. 16.—“Large lymphocyte.” *Explant*: one-sixth of primitive streak from near its posterior end from embryo having a short head process. Plasma and embryo extract, smear, methyl alcohol, Giemsa.

FIG. 17.—Phagocytic wandering cell containing ingested remains. *Explant*: posterior one-sixth of primitive streak of embryo having short head process. Plasma and embryo extract, smear, methyl alcohol, Giemsa.

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The Effects on Dogs of Large Doses of Calciferol (Vitamin D).

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A number of investigations have appeared to show some relation between the rise of blood calcium produced by excessive doses of vitamin D and the action of the parathyroid glands. Jones (1926) had shown that the administration of cod liver oil to dogs in large doses, for some weeks before removal of the parathyroids, prevented the usual onset of tetany and increased the length of survival, whereas similar treatment with the oil was ineffective after the glands had been removed. The result was in line with a suggestion which had already been made with reference to irradiation [Block and Faber (1925)] that the effects of excessive doses of the vitamin D on blood calcium were not produced by a direct action, but by stimulation of excessive output of the parathyroid hormone. Greenwald and Gross (1929) explained Jones' results in this way, and showed that even 300–400 mgm. of irradiated ergosterol did not definitely relieve tetany or raise the blood calcium, unless it was supplemented by adding calcium to the diet. The experiments of Hess and his co-workers [Hess and Lewis (1928); Hess, Weinstock and Rivkin (1929, 1930); Hess, Benjamin and Gross (1931)] seemed to point, on the whole, in the same direction. Their results showed that larger doses of irradiated ergosterol are needed to produce a rise of blood-calcium on a calcium-free than on normal diets, and that still larger doses are necessary if the parathyroids have been removed in addition.

These and other similar investigations appeared to show that the effects of vitamin D, when given in doses large enough to cause a toxic hypercalcaemia, may be produced in several ways: (1) when there is plenty of calcium in the food, large doses of the vitamin promote excessive absorption of calcium from the alimentary canal, or alternatively, diminished re-excretion into the bowel [Taylor and Weld (1932)], just as therapeutic doses bring back a defective absorption to within the normal range. (2) When calcium is deficient in or absent from the food, larger doses of the vitamin can still cause a hypercalcaemia by withdrawal of calcium from the tissues. The work of Kreitmair and Hintzelmann (1928), Baumgartner, King and Page (1929), György (1930),

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Harris and Innes (1931), and Shelling (1932, 2) has shown that under such conditions calcium is withdrawn from the bones. (3) When the parathyroids are removed from an animal on a calcium-free diet, very large doses of the vitamin can still produce hypercalcaemia [Jones, Rapoport and Hodes (1930); Shelling (1932, 1)].

The resemblance between the effects of vitamin D in great excess, on a diet poor in calcium, and those produced by excess of the parathyroid hormone—hypercalcaemia with decalcification of the bones—naturally reinforced the suggestion that the effects of the vitamin under such conditions were produced indirectly, by stimulating output of the hormone. The hypercalcaemia still produced by the vitamin, in adequate doses, after removal of the parathyroids by the ordinary operation, would in that case have to be attributed to presence of accessory masses of parathyroid tissue, which had escaped removal. This is the view adopted by Taylor, Weld, Branion and Kay (1931), as the result of an extensive series of experiments. They drew attention to the close similarity between the symptoms produced in dogs by toxic doses of irradiated ergosterol and of the parathyroid hormone respectively, and between the appearance of the different organs—gastrointestinal mucous membrane, lungs, etc.—in animals dying from the two kinds of intoxication. They further drew attention to the parallelism between the effects of overdosage with vitamin D, and with parathormone respectively, in a range of different species. In dogs subjected to simple removal of the parathyroids with the thyroid gland, the resulting tetany was promptly relieved by administration of a large dose of irradiated ergosterol, and repeated administration led to the ordinary symptoms of overdosage. When, in addition, all tissue was removed which might harbour aberrant parathyroids, from the angle of the jaw to the sternum on both sides, the tetany was much more resistant to treatment with vitamin overdosage, and the latter had to be continued much longer before, eventually, the animals succumbed with the usual signs and symptoms produced by such excess. They regarded these results as confirming the view that excess of the vitamin acts through stimulation of the parathyroids, on the supposition that, after the most complete dissection of the neck, aberrant parathyroid tissue, embedded in the thymus or other mediastinal tissue, might still be left. The assumption seems to be involved, that a remnant of parathyroid tissue, inadequate to prevent a fatal tetany without further treatment, and incapable even of removing tetany under the stimulus of the vitamin until a very large excess has been administered, is nevertheless able, under continued stimulation, to produce death from parathyroid excess. The

conception seems to us to present difficulties. It should be noted, further, that Taylor and his co-workers did not begin to administer the vitamin until tetany had appeared, and then gave it by the mouth, in spite of difficulties due to trismus and vomiting. Under such conditions there might be doubt as to the effective absorption of the doses administered.

There has been some doubt as to whether the toxic effects of overdosage with the mixed irradiation product are due to excess of the vitamin itself, or to other derivatives formed from ergosterol at the same time. Hoyle (1930) attributed the effects to toxic impurities, and Holtz and Schreiber (1930), Windaus (1930), Windaus and Aubagen (1931) have shown that the mixed product can be so treated as to give a preparation devoid of the therapeutic action, but still toxic in large doses; though the evidence is not clear as to whether the toxic constituent pre-existed as such in the antirachitic mixture, or was produced by further change of the vitamin. The question has been at least partly answered by the isolation of the pure crystalline vitamin D, named calciferol in England [Askew, Bourdillon *et al.* (1931)] and vitamin D₂ in Germany [Windaus *et al.* (1931)]. Both sets of observers found that the pure substance showed, in excessive doses, a toxicity for rats and mice proportionate to its antirachitic activity in small doses. It was to be expected that the same would hold good for the toxic effects in dogs. The availability of pure calciferol enabled this point to be settled. Since the pure substance could be prepared with relative ease in a state of high dispersion in a watery medium, suitable for intravenous injection, it also afforded a better opportunity of testing the question, whether the condition of the gastrointestinal mucous membrane, seen after fatal doses, is due to local action, or is entirely secondary to the hypercalcaemia.

The following short series of experiments on dogs has been made to obtain further evidence on these various points. The calciferol used was generously placed at our disposal by the British Drug Houses, Ltd.

Methods.

For determinations of calcium and phosphorus, blood was obtained either by allowing it to drop from a puncture in the lateral ear vein, or with a syringe from the external saphena vein, which was also used for injections. Calcium was determined by Clark and Collip's (1925) modification of the Kramer-Tisdall method, and phosphorus by that of Fiske and Subbarow (1925). For intra-

venous injection a 3 per cent. solution of calciferol in alcohol was added, drop by drop with vigorous stirring, in the proportion of 3 c.c. to 9 c.c. of sterile dog serum. An opalescent dispersion was thus produced, which was warmed to body temperature and slowly injected. Such injections were usually tolerated without any immediate symptoms, but one dog vomited shortly after an injection. If such a dispersion is kept for days in the cold, the calciferol ultimately separates in small crystals, which cause intravascular clotting if injected. For administration by the mouth a 5 per cent. solution in olive oil was used, the small measured volume being delivered from a syringe on to the back of the tongue and completely swallowed.

Results.

1. *Normal Dogs.*—The object of these experiments was to determine whether pure calciferol would produce the toxic effects characteristic of excessive dosage with vitamin D in dogs, to obtain an idea of the dose required to produce these effects, and to afford a general comparison between the effects of intravenous and oral administration. No attempt was made to determine a precise lethal dose by either method. This would have required a large number of experiments, and the total quantity needed to produce a lethal effect would probably have been different with administration in a single dose, from that required with divided dosage. No special measures were taken to control the diet, the dogs receiving the ordinary ration of meat and biscuits. The relation between dietary calcium and dosage had been sufficiently studied by earlier observers with impure irradiation products. For our simple objects a few experiments gave the information sought. To avoid large numbers we have expressed the doses in milligrammes of calciferol. These can be converted into international units of vitamin D activity on the basis of 40,000 units per milligramme.

Dog 1. 8·9 kgm.

February 1 . . 50 mgm. calciferol given intravenously.

The only symptoms observed were some lassitude and loss of appetite on February 3. Otherwise the dog remained lively and ate well. The stools, however, became loose and remained so, with occasional blood, for 2 weeks after the injection. The serum-calcium, initially 10 mgm. per cent., showed only the relatively small rise to 12·6 mgm. per cent. for a few days.

Dog 2. 8 kgm.

- February 4 .. 100 mgm. calciferol intravenously. Serum calcium, 11·5 mgm. per cent.
- February 9 .. Dog, which had not been obviously abnormal, refused food and lost activity. Weight 6·9 kgm. Serum calcium, 18·3 mgm. per cent.
- February 10 .. Serum calcium, 19·5 mgm. per cent.
- February 11 .. Apparent improvement of condition and appetite. Serum calcium, 18·7 mgm. per cent.
- February 12 .. Dog had vomited in the night and showed renewed anorexia and lassitude. Serum calcium, 18·5 mgm. per cent.
- February 13 .. Dog becoming weak. Weight 5·95 kgm. Killed with chloroform. Serum calcium, 17·5 mgm. per cent.

Post-mortem.—Nothing definitely abnormal in lungs or liver. Gastro-intestinal mucosa mildly congested. An ulcerative condition of the gums, which must have been present undetected at the beginning of the experiment, was evidently progressive. The rapid loss of weight and condition must, however, be associated with the high serum calcium, which rose to 19·5 mgm. per cent. on February 10, and fell slowly to 17·5 mgm. The dose appeared to be near the lethal limit for a dog of this size.

Dog 3. 7·8 kgm.

- February 8 .. 50 mgm. calciferol intravenously. Serum calcium, 10·7 mgm. per cent.
- February 9 .. 50 mgm. calciferol intravenously. Serum calcium, 12·9 mgm. per cent.
- February 10 .. 50 mgm. calciferol intravenously. Serum calcium, 18·1 mgm. per cent.

Total calciferol, 150 mgm. over 2 days.

The serum calcium rose sharply and had reached 20·8 mgm. per cent. on February 11. By this time the dog showed the usual symptoms of lassitude, weakness, anorexia and passage of loose faeces with blood. At 5 p.m. on this day the dog, which was obviously going to die, was killed under an anaesthetic.

Post-mortem.—Gastro-intestinal mucosa and lungs distinctly, but not severely, congested. Serum calcium, 20·5 mgm. per cent.

These three experiments showed that the lethal dose of calciferol, with a single intravenous injection, or a few injections over 2 days, was in the neighbourhood of 100–150 mgm. for these dogs. The dose probably varies with the age of the dog, and it is unlikely that it can be expressed merely in terms of body weight. With this reservation, the lethal dose can be taken as about 12–20 mgm. per kilogramme. The following experiment shows the effect of a single much larger dose.

Dog 4. 8·7 kgm.

February 8 . . . 400 mgm. calciferol intravenously. Serum calcium, 10·5 mgm. per cent.

February 9 . . Apparently normal. Serum calcium, 15·5 mgm. per cent.

February 10 . . Less active, appetite poor. Weight 8·35 kgm. Serum calcium, 17·2 mgm. per cent.

February 11 . . Has vomited and passed blood *per anum* during the night. Very weak. Weight 7·65 kgm. Dies at 10·45 a.m.

Post-mortem (immediate).—Serum calcium, 19·5 mgm. per cent. Mucosa of stomach and intestine swollen and intensely congested with hæmorrhage into the lumen. Liver much congested; lungs less so. The blood was very viscous, and was found to contain 60 per cent. of corpuscles by volume; Hb = 157 per cent. by the human hæmoglobinometer (Haldane).

The next experiment shows the effect of repeated doses by the mouth.

Dog 5. 15 kgm.

February 8 . . 50 mgm. calciferol by mouth. Serum calcium, 10·6 mgm. per cent.

February 9 . . 50 mgm. + 60 mgm. by mouth. Serum calcium, 13·5 mgm. per cent.

February 10 . . 80 mgm. by mouth. Serum calcium, 19·3 mgm. per cent.

February 11 . . Serum calcium, 18·2 mgm. per cent.

Total, 240 mgm. over 2 days.

This dog showed slighter symptoms of intoxication than those treated by intravenous injections, though it vomited on occasion. The serum calcium, however, had risen to 19·3 per cent. already on February 10, and the dog died early on February 12. The usual post-mortem appearances—congestion of the alimentary mucosa and of the lungs—were present in moderate degree.

The dose, it will be seen, amounted to 16 mgm. per kilogramme.

These experiments showed that pure calciferol, in excessive doses, produces the typical fatal hypercalcaemia in dogs produced by the mixed irradiation product. Intravenous injection appeared to be neither more nor less effective than oral administration in producing the fatal intoxication. The rapidity of onset of the symptoms and of the fatal termination was not noticeably different by the two methods. Dog 3 and dog 5, which probably received not much more than the fatal quantity, given over 2 days in each case, but intravenously in the one case and by mouth in the other, both died on the fourth day. It should be noted, in particular, that effects on the alimentary mucosa were as severe with intravenous as with oral administration; the congestion was, therefore, secondary to a systemic condition, presumably associated with the hypercalcaemia, and not to a local action on the mucosa.

Effects after Parathyroid Extirpation.

Here again only a few experiments have been performed. Our object was to test the evidence which has been supposed to support the view that excessive doses of the vitamin produce their effects through the parathyroids. For this purpose it seemed to us undesirable to allow the effects of deprivation to appear, before the administration of calciferol was started. If calciferol acted by stimulating the parathyroids, it should fail to act when these glands had been removed, even though there had not been time for hypercalcaemia and tetany to appear. On the other hand, if the hypercalcaemia due to calciferol had begun to appear, its progress should be arrested by complete extirpation of the parathyroids.

Dog 8. 11·8 kgm.

February 22 . . The dog was given 100 mgm. calciferol by mouth at 2.50 p.m.; it was then at once anaesthetised with ether, and a simple extirpation of both lobes of the thyroid with attached parathyroids was performed aseptically, the operation being completed by 4 p.m. The animal had, therefore, lost its main parathyroid supply long before the initial dose of calciferol had been absorbed, and at least a day before any definite rise of the blood calcium would have been expected in a normal dog similarly dosed. Serum calcium at end of operation, 11·6 mgm. per cent.

- February 23 .. 10 a.m.—25 mgm. calciferol by mouth.
5 p.m.—25 mgm. calciferol by mouth.
Serum calcium, 13.2 mgm. per cent. Condition apparently normal.
- February 24 .. 10 a.m.—25 mgm. by mouth.
5 p.m.—25 mgm. by mouth.
Total, 200 mgm. or 17 mgm. per kilogramme. Serum calcium, 16.8 mgm. per cent. Dog seems apathetic.
- February 25 .. 10 a.m.—Very weak and lethargic. Has vomited much during night. Serum calcium, 17.4 mgm. per cent.
12 noon.—Dies. Serum calcium, 16.2 mgm. per cent.

Post-mortem.—Lungs and alimentary mucosa mildly congested. Some free blood in intestinal lumen.

In the onset of symptoms, the rapidity of their fatal termination, and in the post-mortem appearances, this dog clearly showed no significant difference from those receiving similar doses of calciferol without removal of the parathyroids. The blood calcium also rose as promptly; the only apparent difference being that the level reached at the fatal termination (16–17 per cent.) was lower than in the unoperated dogs (19–20 per cent.). On the other hand, the result was not seriously different from those which Taylor and his colleagues obtained with this limited operation, and attributed to the presence of aberrant parathyroids. It was necessary for us to try the effect of a more complete exclusion. Two experiments were therefore made, in which the complete dissection of the neck, from the surface of the submaxillary gland above to the sternum below, was carried out, in addition to wide excision of the thyroid and attached parathyroids. Every suspicious fragment of tissue was removed, in accordance with the method of the Canadian authors, the lymphatic glands in relation to the submaxillary glands being excised as a further precaution.

Dog 9. 14.2 kgm.

In this experiment a large dose of calciferol was given on the day *preceding* the operation. We wished to observe whether the parathyroidectomy would interfere with, or delay, the hypercalcaemia.

- February 25 .. Serum calcium, 12.3 mgm. per cent.
10 a.m.—100 mgm. calciferol by mouth.
5 p.m. 50 mgm. calciferol by mouth.

February 26 .. 10 a.m.—Serum calcium, 15·3 mgm. per cent.
 11 a.m.—Anæsthesia with Dial intraperitoneally, supplemented by ether as required.
 1 p.m.—Aseptic removal of all parathyroid tissue in the neck completed.
 7 p.m.—40 mgm. calciferol by mouth.
 Total, 190 mgm. (13·4 mgm. per kilogramme) over 33 hours.
 500 c.c. warm milk with 10 gm. sugar by stomach tube at intervals after the operation.

February 27 .. 6.30 a.m.—Found dead.

Post-mortem.—Alimentary mucosa severely congested. Serum calcium, 15·3 mgm. per cent.

The operation does appear to have stopped the upward course of the calcium curve, but not to have delayed the fatal outcome, with typical effect on the mucosa.

Dog 10. 13·2 kgm.

In this case the first dose of calciferol was given, intravenously, immediately after the operation.

February 29 .. 10.30 a.m.—Serum calcium 12·2 mgm. per cent.
 10.45 a.m.—12.15 p.m.—Complete excision from neck of thyroid and all tissue possibly harbouring parathyroids, under Dial and ether anæsthesia.
 12.30 p.m.—100 mgm. calciferol intravenously.
 6 p.m.—Serum calcium, 8·8 per cent.
 3 raw eggs and 10 gm. sugar by stomach tube, with 300 c.c. of water.

March 1 .. 11 a.m.—Serum calcium 12·1 mgm. per cent.
 50 mgm. calciferol intravenously.
 Dog still drowsy from Dial.
 5 p.m.—Takes 30 gm. fish and 50 gm. meat and water freely. Serum calcium, 12·8 mgm. per cent.
 12 midnight.—Serum calcium, 15·5 mgm. per cent.

- March 2 .. 11 a.m.—Serum calcium, 15·6 mgm. per cent. Weakness and anorexia.
50 mgm. calciferol by mouth.
Total 200 mgm. or 15·2 mgm. per kilogram.
4 p.m.—Serum calcium, 16·5 mgm. per cent.
11 p.m.—Serum calcium, 17·8 mgm. per cent.
- March 3 .. Diarrhœa, with blood. Much weaker. Food given by tube immediately vomited.
10 a.m.—Serum calcium, 19·1 mgm. per cent.
4 p.m.—Serum calcium, 19·4 mgm. per cent.
11.30 p.m.—Serum calcium, 17·2 mgm. per cent.
Dies during night. Severe congestion and consolidation of the lungs, and pronounced hæmorrhagic congestion of whole gastrointestinal mucosa.

It will be seen that in this dog the blood calcium had fallen from the rather high normal of 12·2 mgm. per cent. to 8·8 mgm. per cent. in the 5½ hours which followed the completion of the parathyroidectomy, in spite of the fact that 100 mgm. of calciferol were injected at the end of the operation. The effect of parathyroid deprivation was, therefore, initially ahead of that of calciferol. By the next morning the blood calcium had returned to the normal, and thenceforward, with further doses of calciferol up to the normal fatal limit, rose continuously, death occurring on the fourth day with symptoms and post-mortem findings quite similar to those seen in the normal animal receiving a similar dosage.

The possibility was considered that the use of a stable anæsthetic, such as Dial, might complicate the result, by reducing the resistance of the tissues to the calciferol action, and thereby accelerating its effect. One further experiment was therefore made, in which all possible parathyroid tissue, accessible without opening the chest, was removed by an operation similar to the foregoing, but under pure ether anæsthesia.

Dog 12. 14 kgm.

- July 25 .. 10.30 a.m.—Blood from saphena vein—serum calcium, 13 mgm. per cent.
10.45. a.m.—Operation started under full ether anæsthesia. Thyroid with attached parathyroids, lymphatic glands, and all tissue possibly harbouring accessory parathyroids

completely removed, from the submaxillary glands to the pleura.

11.45 a.m.—Operation completed and suturing begun.

12 noon.—150 mgm. of calciferol intravenously.

12.15 p.m.—Dog begins to recover from ether.

6.30 p.m.—Serum calcium, 9 mgm. per cent.

July 26 .. Animal fairly normal and active.

10.30 a.m.—Drank 150 c.c. of milk in which 120 mgm. of calciferol were emulsified.

10.45 a.m.—Serum calcium, 11.5 mgm. per cent. Drank 200 c.c. milk with 20 gm. glucose.

12 noon—2 p.m.—Vomited hair and mucus, with a little milk clot.

2.30 p.m.—Ate a little meat, but vomited it shortly afterwards.

5.30 p.m.—40 mgm. of calciferol in oil placed on the back of the tongue and swallowed.

Total administered, 310 mgm.

July 27 .. Dog fairly strong and active. Respiration somewhat laboured. Had drunk water during the night, and passed loose fæces, but had not eaten.

10.45 a.m.—Serum calcium, 16.2 mgm. per cent.

11 a.m.—1 p.m.—Vomited clear, bile stained fluid.

1-2 p.m.—Passed loose fæces.

5.30 p.m.—Serum calcium, 17.6 mgm. per cent. Refused offer of raw meat.

10.30 p.m.—Somewhat weaker. Serum calcium, 17.7 mgm. per cent.

July 28 .. Dog still weaker. As it seemed likely to die before the next morning, it was killed under ether at 2.30 p.m.

Post-mortem.—Gastric mucosa slightly congested, that of the intestine strongly so. Lungs distinctly but not extremely congested. Serum calcium, 19.6 mgm. per cent.

It was clear then, that the absence of the stable anæsthetic did not weaken the toxic action. The serum calcium had risen to nearly a lethal value in 3 days, and the animal would have died within a further 24 hours, if the intoxication had been allowed to follow its course. The total quantity administered was 310 mgm. of calciferol, but it may be supposed that at least

30 mgm. of this were lost by vomiting, so that not more than 280 mgm., or 20 mgm. per kilogram, are likely to have been absorbed.

Several attempts were made to ensure an even more certainly complete removal of the parathyroids by proceeding, after the radical removal in the neck, to open the chest by splitting the sternum and remove the thymus and attached tissue completely. In two such cases it was necessary to insert a tracheal tube and perform artificial respiration, since the thymus could not be removed completely without opening the pleuræ; and in these cases, although the chest was successfully closed, with re-expansion of the lungs and effective resumption of normal respiration, death occurred before recovery from the stable anæsthetic (Dial), and before the result could be attributed to the calciferol. In these cases the lungs were found to be massively congested. It seemed possible that their exposure in operation had rendered them abnormally liable to the action of the calciferol, but the experiments could not be regarded as having clear evidential value. In a third dog we were fortunate in finding an anatomical relationship which enabled the thymus to be completely removed after splitting the upper part of the sternum, without opening the pleuræ or interfering with the natural respiration.

Dog 11. 10 kgm.

- April 27 .. 11 a.m.—4 c.c. Dial intraperitoneally.
2 p.m.—Serum calcium, 11.3 mgm. per cent. Operation under Dial anæsthesia, with ether as required.
3.10 p.m.—Completion of radical extirpation in neck.
1 c.c. Dial intraperitoneally.
3.15 p.m.—Sternum split and thymus completely removed without opening pleuræ.
4 p.m.—Removal of thymus complete. Suturing begun.
4.20 p.m.—150 mgm. calciferol intravenously.
5.30 p.m.—20 gm. glucose in 250 c.c. water, and 100 mgm. calciferol, given by stomach-tube.
Total calciferol, 250 mgm. or 25 mgm. per kilogram.
- April 28 .. 10.30 a.m.—Dog conscious, but drowsy. Drinks water.
Serum calcium, 12.9 mgm. per cent.
12 noon and 2.20 p.m.—Takes two raw eggs beaten up with 20 gm. glucose and water.
4.15 p.m.—Loose motion.
4.45 p.m.—Serum calcium, 15.2 mgm. per cent.

April 29 .. 7.30 a.m.—Becoming very weak. Has vomited bile-stained fluid.

8.30 a.m.—Vomits.

9.10 a.m.—Serum calcium, 15.0 mgm. per cent.

1.30 p.m.—Dies.

Post-mortem.—Large clot in the right side of the heart. Lungs intensely congested, with fluid in the pleural cavities. Mucous membrane of the stomach shows the typical intense congestion. No remains of the thymus could be found.

This dog had received a total quantity of calciferol somewhat in excess of the lethal dose for a normal dog. There is no reason, however, to suppose that a smaller quantity would not have been effective, and the dog died, with the characteristic symptoms, in less than the usual period after the beginning of the administration—2 days instead of 4.

Discussion.

The only difficulty presented by these results is that of reconciling them with those recorded by Taylor, Weld, Branion and Kay (1931), and with the view which they based on them, attributing the toxic effects of vitamin D in excess to stimulation of parathyroid activity. Our dogs, after removal of the parathyroids as complete as that carried out by these authors, and in one case possibly even more complete, succumbed to calciferol intoxication not less readily than normal dogs. The difference in result is doubtless due to difference in procedure. Taylor and his colleagues waited for the full development of tetany before beginning the administration of irradiated ergosterol. This would have the possible advantage of providing direct evidence of the success of the removal. On the other hand, it would have the effect of producing a low starting level of blood calcium, with a corresponding delay in reaching a toxically high level after treatment was begun. We proceeded on the assumption that, if calciferol acted through the parathyroids, resistance to its toxic action ought to be produced immediately by removal of those glands, and without waiting for the symptoms of deprivation to appear. In two cases (dogs 10 and 12), it will be seen that a determination of serum calcium within 6 hours of the extirpation, and of the immediately following injection of calciferol, showed the fall characteristic of parathyroid loss, with calciferol already circulating. The effect of parathyroidectomy was quicker in onset than the opposite effect of calciferol, but the latter overtook it and prevailed. The only

difference which the absence of the parathyroids appeared to make to the toxic action of calciferol in our series, was to lower somewhat the level of blood calcium at which the action became fatal. The effect, when it was obvious, was equivalent to lowering the base-line from which the hypercalcaemia started, without altering the toxic action which accompanied it. We have not sufficient data to justify a theory of this effect; but such a result would be expected if the action of calciferol were to cause the appearance of abnormal concentrations of calcium in the alimentary mucosa and the lungs as well as in the blood, and if the removal of the parathyroids prevented the full rise of blood calcium, without similarly protecting the lungs and the alimentary mucosa from the accumulation of a toxic excess. In any case, such effects as we have obtained would appear to be fully explained on the supposition that removal of the parathyroids and excess of calciferol affect the blood calcium in opposite directions, without having recourse to the assumption that calciferol acts by stimulating the parathyroids.

Our results, and their differences from those of Taylor and his co-workers, would be rather more easily reconciled with a hypothesis that calciferol acts, not by stimulating excessive secretion of the parathyroid hormone, but by rendering the organism abnormally responsive to the action of what is already in circulation. On this view, calciferol administered immediately after removal of the glands, as in our experiments, would indeed be expected to produce its toxic action more readily than in those of the Toronto workers, who waited for the appearance of severe symptoms before beginning the treatment. There are details in the evidence, however, which are difficult to reconcile with this supposition. It would be expected, for example, that calciferol, administered directly after extirpation of the parathyroids, would show its full effect at once and a declining effect as the hormone became deficient. We find, on the contrary, that the injection of calciferol has no immediate effect on the fall of blood calcium following the extirpation, and only begins to show its action, and then a practically normal one, some hours later, when the fall of blood calcium due to hormone deficiency would be already well in evidence. If the administration of the vitamin is delayed until the symptoms of hormone deficiency are well established, it might be expected quickly to show its sensitising effect to the traces of hormone still remaining, and then to fail of further action as the last traces become exhausted. Taylor and his colleagues found, on the contrary, a delayed effect of the vitamin under these conditions, but one which progressed with continued administration, to death with the characteristic symptoms of vitamin excess.

It appears to us, accordingly, that neither of these alternative views, of a direct connection between calciferol intoxication and the action of the parathyroid hormone, can properly be reconciled with the facts. The similarity of the symptoms and of the post-mortem appearances, produced by a fatal excess of either, seems to be adequately explained by the fact that a sufficient excess of calcium in the blood and tissues, whatever the cause, even if it is produced by continued infusion of calcium salts, has this characteristic result, as demonstrated by Collip (1926). Such modifications in the toxic effects of calciferol as parathyroidectomy produces—any delay in their appearance, increase of necessary dosage, lowering of the level of blood calcium at which death occurs—seem to us to be adequately explained on the simpler supposition that the effects of calciferol and of parathyroidectomy on the concentration of calcium ions in the blood, are in opposite directions, but not connected by any causal relationship.

The fact that calciferol possesses the same toxic action, in excessive doses, as the crude product of irradiating ergosterol, may seem difficult to reconcile with the evidence that the mixed irradiation product can be deprived of its therapeutic action and yet retain the toxic action in large doses. The structure of calciferol is not sufficiently known to justify any definite theory; but the suggestion of Windaus (1930), that the therapeutic action of small and the toxic action of large doses are due to different groups or linkages in the molecule, and that a substance is produced, by appropriate treatment of the irradiation product, having the structural feature which conditions the toxic but lacking that which conditions the therapeutic effect, appears to meet the facts already known.

We gladly acknowledge valuable help received from our colleague Mr. T. A. Webster, in carrying out these experiments and considering their results.

Summary.

The following are our main results and conclusions:—

- (1) The pure, crystalline vitamin D, calciferol, has, in excessive doses, the characteristic toxic action on dogs of the crude product of irradiation of ergosterol.
- (2) The toxic action is produced by intravenous injection as well as by oral administration. The congestion of the alimentary mucosa, produced by a fatal dose, is equally pronounced with either method of administration.
- (3) Complete parathyroidectomy does not prevent, or significantly hinder,

the fatal intoxication produced by large doses of calciferol. At most it lowers the level of concentration reached by the blood calcium before death.

(4) The results lend no support to the suggestion that vitamin D in excessive doses acts by promoting secretion of the parathyroid hormone, or by rendering the organism more responsive to its action.

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